Inflammatory airway disease in horses: The association between bronchoalveolar lavage cytology and pulmonary function testing

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I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution.

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Abstract

Inflammatory airway disease (IAD) describes a condition of non-septic inflammation of the lower airways in horses. The disease occurs principally in adult horses and has an apparent worldwide distribution. The most common clinical signs of IAD include poor athletic performance, cough, and/or increased tracheobronchial secretions. Inconsistencies in disease definition, sampling methods and laboratory techniques have limited comparisons between studies.

Essential criteria for diagnosis of IAD, as stated by the 2007 ACVIM consensus statement, include documentation of non-septic inflammation or pulmonary dysfunction based on evidence of lower airway obstruction, airway hyper-responsiveness, or impaired blood gas exchange at rest or during exercise. A definitive diagnosis is currently based on bronchoalveolar lavage fluid (BALF) cytology and/or pulmonary function testing (PFT).

The correlation between BALF cytology and pulmonary function testing (PFT) has been poorly defined. The primary aim of this study was to characterise the relationship between BALF cytology and PFT with histamine bronchoprovocation methods in a population of sedentary asymptomatic horses. The principal hypothesis was that a strong association exists between these two diagnostic methods.

On the basis of BALF cytology the majority of horses in this study had lower airway inflammation as defined by published criteria. The study thus highlights that normal values for cell proportions in BALF might vary between populations of horses. Despite an obvious overlap between inflammatory BALF cytological profiles and airway hyperresponsiveness, no statistical association between these two diagnostic methods was found in this population of horses.

The secondary aim was to assess the reliability of the Open Pleth™ PFT system Acceptable reliability {ICC: 0.655 (95% CI: 0.098, 0.952; significance: 0.011)}) was demonstrated using the Flowmetrics Plethysmography™ system with histamine bronchoprovocation.

In conclusion, airway inflammation and airway hyperreactivity are loosely related to each other in this population of horses. The presence of inflammatory cells does not necessarily predict airway hyperresponsiveness on the basis of histamine bronchoprovocation. Likewise, airway hyperresponsiveness can occur in the absence of a BALF inflammatory profile. Further investigation of other potential factors such as inherited abnormalities of smooth muscle contractility, airway wall remodelling, autonomic dysfunction, and the presence of inflammatory cell mediators in bronchoalveolar lavage fluid are warranted.

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"Permanence, perseverance and persistence in spite of all obstacles, discouragements, and impossibilities: It is this, that in all things distinguishes the strong soul from the weak." – *Thomas Carlyle*

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Abbreviations

ACVIM	American College of Veterinary Internal Medicine
AHR	Airway hyper-reactivity
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
CDE	Corn dust extract
C_{dyn}	Dynamic compliance
DDSP	Dorsal displacement of soft palate
EHV	Equine herpes virus
EIPH	Exercise induced pulmonary haemorrhage
FE	Forced expiration
FEFV	Forced expiration flow volume
FOT	Forced oscillation techniques
FP	Flowmetric plethysmography
HIPT	Histamine inhalation provocation test
IAD	Inflammatory airway disease
ICC	Intra-class correlation coefficient
IOS	Impulse oscillometry system
IL	Interleukin
LPS	Lipopolysaccharide
µg	Microgram
mg	Milligram
mL	Millilitre
PC_{35}	35% provoking concentration

PFT	Pulmonary function test
RAO	Recurrent airway obstruction
RIP	Respiratory inductance plethysmograph
RUP	Respiratory ultrasonographic plethysmograph
SAID	Small airway inflammatory disease
TCC	Total cell count
TNF	Tumour necrosis factor
TW	Tracheal wash
URT	Upper respiratory tract

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Introduction

Inflammatory airway disease (IAD) describes a condition of non-septic inflammation of the lower airways in horses. Essential criteria for diagnosis of IAD as stated by the 2007 ACVIM consensus statement include documentation of either non-septic inflammation or pulmonary dysfunction based on evidence of lower airway obstruction, airway hyper-responsiveness, or impaired blood gas exchange at rest or during exercise. A clinical diagnosis of IAD is currently based on historical or clinical information with confirmation through bronchoalveolar lavage fluid (BALF) cytology and/or pulmonary function testing (PFT). Studies reporting the association between BALF cytology and pulmonary function testing are limited.

Objectives

1. The primary aim of this thesis was to investigate the association between bronchoalveolar lavage fluid cytology and pulmonary function testing with histamine bronchoprovocation in a population of asymptomatic sedentary horses.
2. The secondary aim was to assess the reliability of the Open Pleth™ system using sedentary horses in Western Australia.

Hypotheses

1. There would be a strong association between lower airway inflammation, as demonstrated by bronchoalveolar lavage cytology, and airway hyperresponsiveness.

2. Flowmetric plethysmography with histamine bronchoprovocation using the Open Pleth™ system is a reliable technique to detect airway hyperresponsiveness.

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

Lower respiratory tract diseases are common conditions seen in equine practice (1). Inflammatory diseases of the lower airways include infectious and parasitic diseases, interstitial pneumonia, recurrent airway obstruction (RAO; heaves) and inflammatory airway disease (IAD). 'Inflammatory airway disease' describes a condition of non-septic inflammation of the lower airways in horses. In 2000 an international workshop on equine airway diseases reported that IAD could have various causes and degrees of severity (2). The syndrome of IAD was defined as a unique phenotype of chronic airway disease, and was distinct from the well-defined chronic lung disease now known as RAO. The latter term was recommended for the mature horse with airway obstruction that is reversed by a change in environment or the use of bronchodilators. A separate syndrome of non-septic lower airway disease, that appeared to be particularly common in young horses, has been recognised for many years. Robinson and colleagues recommended use of the term 'inflammatory airway disease' for these cases (2).

Prior to 2000 there had been confusion over terminology used to describe non-septic inflammation of the lower airway. Past studies have described what is now termed IAD as 'chronic bronchiolitis' (3-5), 'mild bronchiolitis' (6) and small airway inflammatory disease (SAID). In 2007, the American College of Veterinary Internal Medicine (ACVIM) published a consensus statement to define the syndrome of IAD (7). Essential criteria for diagnosis included documentation

of non-septic inflammation, as defined through bronchoalveolar lavage fluid (BALF) cytology or pulmonary dysfunction based on evidence of lower airway obstruction, airway hyper-responsiveness (AHR), or impaired blood gas exchange at rest or during exercise (7).

2.2 Epidemiology of IAD

Inflammatory airway disease is a disease of adult horses that has an apparent worldwide distribution. A very wide range (1.8% to 80%) in disease prevalence has been reported (4,8-11). Comparison between studies has been extremely difficult due to inconsistencies in the definition of the disease and different sampling methods and laboratory techniques used in disease identification. This, together with poor disease recognition by owners and veterinarians, is likely to have resulted in the wide range in prevalence.

Historically cases of IAD were likely under-represented, as illustrated in a North American study undertaken in 1991, where horses were presented for evaluation of poor performance (4). Although 22% of these cases were diagnosed with respiratory problems, only 1.8% of horses were diagnosed with 'sub-clinical chronic bronchiolitis', now termed IAD (2). The low disease prevalence in that study was likely due to a lack of appropriate methods used in diagnosis of the disease.

Much of our understanding of IAD is derived from studies that have used tracheal mucus and tracheal wash (TW) cytology to diagnose "airway

inflammation". Studies have reported a similar worldwide prevalence of "airway inflammation", as defined by both increased tracheal mucus and a greater than expected proportion of neutrophils in tracheal wash cytology, of 13.9% in the UK (11) and 17.3% in the USA (10). Using the sole criterion of tracheal mucus, a UK study reported an average annual prevalence of 80% in 2-year–old Thoroughbred racehorses (8). In poorly performing horses with cytological evidence of IAD in bronchoalveolar lavage and/or TW fluid a prevalence of 65% has been reported (9). Unfortunately the disease definition criteria using tracheal mucus and TW cytology do not meet the currently accepted global definition of IAD (7). Thus again, knowledge of the epidemiology of IAD is limited by the methods used to establish disease diagnosis.

Likewise in a UK study to quantify the causes of pulmonary disease in adult horses presenting to referral practices in the United Kingdom (1) cases of IAD were likely under-represented through poor case definitions. Horses in the study were categorised into 8 groups dependent on a combination of history, clinical signs and diagnostic testing. Horses with IAD as per today's definition by the ACVIM consensus statement were included in the 'chronic obstructive pulmonary disease', 'infectious' and 'undifferentiated pulmonary disease' groups. It is therefore possible that a very high percentage (78%) of horses in the study would have subsequently been diagnosed with IAD.

There is conflicting evidence regarding the association of IAD (as diagnosed by TW mucus and neutrophilic inflammation) and age, with some studies demonstrating that prevalence decreases with age (8,11-13) and others

reporting the opposite (10,14). Robinson et al. (2006) reported that older horses were at risk for airway neutrophilia (>20% neutrophils), but the increase in percentage neutrophils with age was a consequence to a relative decrease in the number of macrophages rather than an increase in neutrophils (10). As with earlier studies IAD was diagnosed in the aforementioned studies using TW mucus scores and cytology, both of which are no longer accepted diagnostic modalities in the diagnosis of the disease (7).

2.3 Pathophysiology

Exercise-induced hypoxemia is a normal finding in all racehorses during intense exercise (15). Some studies have found that horses with IAD demonstrate a greater degree of hypoxemia than normal horses (3,16-19). The greater degree of hypoxemia is thought to occur due to impaired pulmonary gas exchange.

Two potential hypotheses exist for impairment in gas exchange. The first hypothesis and prevailing opinion from several authors is that IAD can lead to impaired gas exchange and reduced athletic performance (3). Diffusion through tissues is described by Fick's law, which states that the rate of transfer of a gas through a sheet of tissue is proportional to the tissue area and the difference in gas partial pressure between the two sides, and inversely proportional to the tissue thickness (20). In horses with IAD, the inflammatory response in the lower airway may lead to thickening of the blood gas barrier and therefore limit diffusion of oxygen between the alveolar space and the pulmonary capillary blood thus impairing lung function. This opinion however is not universally

accepted. Nyman et al. (1999) reported that Standardbred racehorses with IAD, diagnosed on the basis of lung biopsy, could achieve adequate gas exchange during exercise (6). Unfortunately respiratory mechanics using pulmonary function testing were not used to evaluate case or control animals in this study.

A second hypothesis suggests that the presence of tracheal mucus may indirectly affect gas exchange in the distal airway. In a group of horses presented for investigation of poor athletic performance, Durando et al. (2006) demonstrated that the presence of tracheal mucus was associated with abnormal arterial blood gas analysis (21).

2.4 Aetiology

The aetiology of IAD is poorly defined but most authors suggest multiple inciting agents including, but not limited to, viruses (22), bacteria (12,23-26) and environmental loading of the respiratory system, including temperature, humidity, endotoxins, ammonia and dust (23,27-31). These factors may act sequentially or simultaneously in the development of lower airway inflammation (32).

2.4.1 Bacteria

Several epidemiological studies undertaken in the UK and Australia have indicated that bacterial infection plays a major role in lower respiratory tract

disease in racehorses (11,12,22-24,26,33,34). The bacteria most commonly isolated in these studies were *Streptococcus equi* subspecies *zooepidemicus* and *Streptococcus pneumoniae*.

Wood and co-workers reported a decreasing prevalence with age with respect to IAD and recovery of *Streptococcus equi* subspecies *zooepidemicus* and *Streptococcus pneumoniae* from tracheal cultures (11,22). The authors concluded that this finding was due to increased disease resistance associated with the development of immunity to infectious agents (11,22). Other authors have suggested that resolution of bacterial infection may result in residual inflammation of the lower airways with resultant cough (24). A number of studies have suggested that horses with cytological evidence of lower airway inflammation are bacteriologically sterile, supporting the suggestion that there are other causes of lower airway disease in these horses (12, 24).

2.4.2 Viruses

The role of viruses in the development of IAD remains speculative. Unfortunately there are few studies that have investigated the potential role of viruses in the pathogenesis of IAD. There is some evidence that equine herpes viruses (EHV) type 1 and type 4 may be associated with IAD (22,35), but other studies have failed to find any significant association between viral agents and equine lower respiratory tract disease (23,24). These studies are limited by methodology relying on serology to diagnose viral infection and seroconversion may not have yet occurred at the time of investigation.

Bronchoalveolar lavage fluid (BALF) of horses with respiratory viral infection has been shown to contain a large amount of mucosal epithelial cells with numerous free cilia and detached ciliated tufts (36). Similar epithelial cell changes have been associated with the recovery of EHV-2 and EHV-5 DNA in BALF, but not tracheal wash fluid (35).

2.4.3 Upper respiratory tract abnormalities

Conflicting reports demonstrating the association between IAD and upper respiratory tract (URT) abnormalities have been published, with some studies demonstrating no significant association (10,14,18,37) while others demonstrate a strong association (16,21). Courouce-Malblanc et al. (2002) found that horses with upper respiratory tract disease, such as dorsal displacement of the soft palate (DDSP), showed a significantly higher BALF neutrophil percentage compared to control horses (16) and thus suggested that DDSP may be associated with small airway inflammation.

2.4.4 Exercise-Induced Pulmonary Haemorrhage (EIPH)

Histopathological studies have shown the presence of bronchiolitis in areas of lung affected by EIPH (38). Early studies reported an association between EIPH and airway inflammation (25,39-41), although more recent data has not

supported this association. There was no correlation between EIPH and airway inflammation based on airway endoscopic and TW cytological findings in a study by Chapman and others (12). This was further supported by Allen and co-workers (2006) who reported no significant association between EIPH and the presence of tracheal mucus or increased neutrophils in TW or BAL cytology in UK horses (14). It is possible that IAD could contribute to EIPH and there is evidence that blood in the airway may induce a mild but prolonged inflammatory reaction (42). It is not absolutely clear if the neutrophilic response found in this study was the cause or consequence of the haemorrhage.

Repeated administration of blood into the airways can cause alterations that result in an impairment of respiratory mechanics as demonstrated by a decrease in dynamic compliance and increase in pulmonary resistance (43). Aguilera-Tejero and co-workers (1995) suggested that these changes were attributed to the subsequent airway inflammation induced by the presence of blood. In contrast, two later studies did not find any effect on ventilatory measurements by the instillation of blood (44,45). The results of these studies are again difficult to compare due to different methodologies.

2.4.5 Cold weather

Cold air may potentially have a role to play in the pathogenesis of lung disease in horses, including non-septic conditions such as IAD. At rest, inspired air is warmed to body temperature and fully humidified by the upper airway mucosa. When the inspired air is cold or during increased minute ventilation,

conditioning by the upper airway may be insufficient, resulting in cooling and desiccation of lower airway mucosa. The resultant heat and water loss from the lower airways is believed to be the provocative stimulus for exercise-induced bronchoconstriction in humans with pre-existing airway hyper-reactivity.

A study by Davis et al. (2005) found significant increases in pro-inflammatory cytokines (Interleukin (IL) -2, IL-4, IL-5 and IL-6) in the airways of a group of normal horses 5 hours after exercising while breathing cold air (46). In a later study by the same group, increases in pro-inflammatory cytokines IL-1, IL-6 and IL-8 were reported 24h after exercising while breathing cold air in normal horses (47). Davis and co-workers also demonstrated that the proportion of neutrophils in BALF was significantly higher in these horses when compared to a control group breathing warm air (47). Cold inspired air was also associated with higher respiratory impedance and resistance 48h after exercise challenges (48). Cytological reference values were not obtained at rest prior to exercise in these studies.

An increase in the number of training days lost due to respiratory problems was found during the winter months in a survey of Thoroughbred racehorses in South Africa (49). The average minimum temperature in winter was less than 5^{o}C in this geographic region. It was hypothesised that cold air played a role in the aetiology of lower respiratory tract disease in these horses.

2.4.6 Environmental dusts and gases

Exposure to environmental dusts and gases may also play a significant role in the aetiology, severity and duration of airway inflammation in horses (32). Horses are potentially exposed to high burdens of environmental particulates, including microorganisms and potential allergens from feed and bedding within the stable, as well as particulates from the external environment. Direct exposure to stable dust, such as that found in hay (27,32) and bedding (23) can induce airway inflammation. Horses that consume hay, especially from round bales, demonstrate increased number of neutrophils in lower airway secretions (10, 27) and the overall dust content of hay and concentrations of dust in the breathing zone can exceed 20 mg/m^3 of air when horses eat from bales (31). Burrell et al. (1996) reported that horses bedded on paper were less likely to develop lower airway disease than those bedded on straw in loose boxes (23). In a group of horses with poor performance and BALF neutrophilia, diagnosed with IAD, positive clinical responses were found when environmental changes were made such as alterations in bedding materials, feeding practices and stable ventilation (50).

In a US study, significant associations were found between the presence of visible accumulations of tracheal mucus and the concentration and size of particles within a stable (51). A UK study found that the mean duration of IAD incidents was significantly higher for horses kept in an environment where dust loading was higher and ventilation was poor (23). The concentration and number of particles throughout stables has been shown to vary with month, time

of day, stable and location of stall within a stable (51). Stalls in the centre of the barn or near areas of high traffic, such as doorways or adjacent to busy roads were shown to be at greatest risk of high particulate counts in a study by Millerick-May and co-workers. Insufficient ventilation within enclosed brick stables also increased the risk of exposure to particulate matter and development of excess tracheal mucus (51). The increased challenges to the respiratory tract from microorganisms or allergens may affect the clearance of pathogens and change local or systemic immune resistance thus resulting in inflammation (52).

Endotoxin, a major pro-inflammatory glycolipid component of gram-negative bacteria, is a constituent of airborne dusts and has been shown to play an important role in lower airway inflammation in horses (32) and in other species (53,54). Inhalation of endotoxin results in a dose-dependent increase in the concentration of tumour necrosis factor (TNF) in BAL fluid in rats (53). Aerosolized TNF induces an influx of neutrophils in BALF and the lipopolysaccharide (LPS)-induced neutrophil influx was partly inhibited by pre-treatment with anti-TNF antibodies in their study (53). Schwartz et al. (1994) demonstrated that endotoxin is a critical component in the development of grain dust-induced inflammation in the lower respiratory tract of humans (54). Collection of BALF 5h after the start of exposure to endotoxin demonstrated a higher concentration of total cells, neutrophils, and TNF-α in BALF after inhalation of corn dust extract (CDE), sterile CDE, or LPS in endotoxin-sensitive mice. The inflammatory response to inhaled grain dust increased as the inhaled concentration of endotoxin increased (54). A significant linear relationship was

found between neutrophilic airway inflammation and exposure to high respirable endotoxin concentrations in an Australian study of young Thoroughbred racehorses (32).

Although stabling of racehorses has been implicated as a risk factor for clinical respiratory disease (32), there is also good evidence that bringing asymptomatic horses of varying age groups from pasture into a conventional stable environment causes an acute neutrophilic airway inflammatory response without consistent clinical signs (27,30,32,55).

2.5 Clinical signs of IAD

The clinical signs attributable to IAD can be subtle. The 2007 ACVIM consensus statement on IAD reported poor athletic performance, cough and increased tracheobronchial secretions (tracheal mucus) as the most common clinical signs of the disease (7). Horses with IAD can be distinguished from horses with RAO in that IAD horses do not exhibit increased respiratory effort at rest (56).

2.5.1 Poor athletic performance

Poor athletic performance is a significant problem in the horse industry. Although poor athletic performance is often subjective, and client expectations frequently outweigh the horse's athletic ability (1), horses can perform poorly

for a multitude of reasons beyond limitations related to ability and fitness (4,9,37,57-59). Many problems are subtle and a number of studies have reported multiple factors contribute to horses performing poorly, with respiratory and musculoskeletal disease being overrepresented (4,9,49,59,60).

While overt respiratory tract disease is a well-recognized cause of reduced performance, the impact of subclinical disease appears to be understated and poorly understood. In a UK study, 65 horses without clinical evidence of pulmonary disease were referred for pulmonary examination because of poor racing performance, pulmonary disorders, including IAD, were attributed to 35 (58.3%) of these cases (1). Comparison between studies is again made difficult due to poor disease recognition, particularly due to the subjectivity of poor performance and subtleness of clinical signs of disease.

A number of investigators have made an association between airway inflammation and exercise intolerance or poor performance (4,50,61). IAD was a common finding in National Hunt horses referred for investigation of poor performance (14) and prevailing opinion from several authors is that IAD can lead to impaired gas exchange and reduced athletic performance (3)(see section 2.3).

With the exception of eventing horses, sport horses typically do not reach maximal cardio-respiratory effort during competition and subclinical airway disease, although present, may not limit performance (55). The apparent high

prevalence of IAD in sport horses (55) casts some doubt on the clinical significance of the disease across all athletic disciplines.

2.5.2 Cough

Cough is an important and common clinical sign of respiratory tract disease in horses, but can reflect both infectious and non-infectious aetiologies. In a study of 300 horses presented to a referral hospital in the United Kingdom, cough was the most common presenting clinical sign of pulmonary disease (62). Cough has been found to be a very specific but insensitive sign of inflammatory changes in the lower airway (10,23,63).

Studies in racehorses have found that cough diminishes with increasing age, suggesting either a development of immunity or tolerance to the aetiological agents, or alternatively that some other component may be more prevalent in younger horses (63). Conversely, Bedenice et al. (2008) found that coughing was significantly more prevalent in horses over 7 years of age and its presence was best characterized by a high relative BALF neutrophil percentage (>5%) and nasal discharge (64). The group hypothesised that this may be due to increased length of exposure to environmental particulate matter in the older horse.

2.5.3 Tracheal mucus

Mucus is produced by specialised cells called goblet cells in the respiratory epithelium and is an important part of the mucociliary escalator that removes

infectious agents and environmental particulates from the lower airways. Mucociliary clearance is normally a very efficient process whereby mucus elimination keeps pace with mucus production, such that the airways contain minimal mucocellular material with low numbers of cells (41). The mucociliary "ladder" is estimated to transport mucus proximally at a rate of 2-8 mL/h in clinically healthy horses (65) and some mucus is expected on visual inspection of the trachea, particularly in racehorses in training (63,66). Mucus may accumulate in the tracheal lumen due to increased production within the lower airways or when mucociliary clearance is impaired (67). Although studies report that the accumulation of mucus in the trachea is a common finding in horses with IAD (14), it is neither sensitive nor specific for IAD, and can be observed in other lower airway diseases (62) and in clinically healthy animals (55), particularly after fast exercise (68). Mucus accumulation also has been shown to develop when RAO-affected horses are stabled and fed hay (69,70).

A tracheal mucus scoring system, where the amount of mucus present was graded between 0 and 5, was validated in 2004 (65). Studies have demonstrated an association between moderate to severe accumulations of tracheal mucus and the two other main clinical signs of IAD - decreased racing performance (9,59,71,72) and coughing (see previous section - 2.5.2)(62,63,69).

The relationship between tracheal mucus accumulation and lower airway inflammation is unclear. A number of studies report no association between the presence of tracheal mucus and BALF cytology (14,55) or tracheal mucus score and cytology of TW fluid or BALF (9,51,59). Some horses in a study by Sanchez et

al. (2005) had excess mucus on endoscopy but normal BALF cytology, and others with no evidence of mucus were found to have an inflammatory BALF (18). In contrast to these studies, mucus accumulation scores have also been shown to correlate well with TW and BALF neutrophil percentages (65).

In light of these discrepancies, it is possible that the activity of the mucus apparatus is independent from lower grade airway inflammation or alternatively, BALF cytology may be too crude a measure of airway inflammation to detect an association.

2.6 Diagnosis

A diagnosis of IAD is currently based on BALF cytology and/or pulmonary function testing.

2.6.1 Blood markers of inflammation

There has been no reported association between blood inflammatory markers and respiratory fluid cytology in horses presented for poor performance evaluation (9) or coughing (73). During exercise testing horses with concurrent DDSP and IAD were found to have higher blood lactate concentrations compared to horses with DDSP alone (57).

2.6.2 Lung biopsy

There is very little data to support a role for lung biopsy in the diagnosis of IAD. Based on the assumption that IAD is a diffuse disease, lung biopsies were evaluated in a study by Persson and Lindberg (1991) to evaluate exercise intolerance in athletic horses (3). This was based on earlier findings that biopsy pathology from horses with RAO was correlated with necropsy findings that included airway wall thickening and inflammation in horses with early clinical signs of obstructive lung disease (74).

Percutaneous lung biopsy is rarely used and should not be regarded as a routine procedure as it carries a significant complication rate (77%)(75). The technique has been described in the standing sedated horse using a 14-gauge 15-cm Tru-Cut biopsy needle or using automated biopsy devices. The preferred site for lung biopsy is the seventh or eighth inter-costal space 8cm above a horizontal line across the elbow joint (76).

Thoracoscopic pulmonary wedge resection allows removal of larger tissue samples. A technique described by Lugo et al. (2002) (77) is reported to be safe and provides good specimens for histology, but is limited to the periphery of the lungs and the sample will therefore only be useful for isolated peripheral lesions or diffuse diseases. Another study by Lugo et al. (2006) demonstrated that a lung biopsy sample obtained from the caudal aspect of the caudal lung lobe is representative of the remainder of the lung in both control and RAO-affected horses (78).

2.6.3 Tracheoscopy

Endoscopy is an important tool used in the investigation of equine upper and lower airway disease. It allows for visual assessment of the trachea (tracheoscopy) to detect the presence of mucus (see section 2.5.3) and subsequent collection of mucocellular material for cytological examination (see section 2.6.4).

There are anecdotal reports describing an association between thickening and blunting of the tracheal septum with IAD (2), but Koch and co-workers (79) did not support this association in a prospective study.

2.6.4 Tracheal wash cytology

Endoscopic examination of the trachea also allows for collection of mucus for cytological examination – a procedure known as a tracheal wash (TW). Tracheal wash samples are representative of both the peripheral and central airways (76). Cytological examination of these samples has been used for several decades as an important adjunct in the diagnosis of diseases of the lower airways (5,41).

Neutrophils are essential elements of the acute inflammatory response, but the clinical relevance of increased neutrophils in TW samples is not clear. A number of studies have indicated that neutrophils usually comprise of less than 20% of TW nucleated cells in clinically normal racehorses (12,63,80). The presence of

more than 20% neutrophils in TW samples has been associated with coughing in young racehorses (34,63) and the isolation of significant bacteria (12).

The relationship between tracheal mucus accumulation and TW cytology is unclear with studies both reporting and denying an association. Neutrophilic inflammation may cause mucus accumulation in horse airways through increased production and secretion of mucins (81) or by altering the physical properties and thus the clearance of mucociliary secretions (82). Durando et al. (2006) found no relationship between endoscopic tracheal mucus and TW cytology (21) whereas other studies have reported that an increased number of neutrophils in TW fluid was associated with mucus accumulation (1,10).

In support of Durando and co-worker's (2006) findings, other studies have reported that only increased tracheal mucus, and not increased TW neutrophils, was associated with poor racing performance in Thoroughbreds (71) and with reduced willingness to perform in show-jumpers and dressage horses (83). This suggests that TW cytology may not consistently represent deeper processes in the lung that produce mucus.

The relationship between tracheal and bronchoalveolar fluid cytology is also unclear, again with studies both reporting and denying any association. A significant correlation was reported between the percentage of neutrophils in TW and BALF samples in horses with IAD (9) and in horses with or without DDSP (57), while others found little or no correlation between neutrophil percentages in TW fluid and BALF (14,32,59). In light of these discrepancies, the

current American College of Veterinary Internal Medicine Consensus Statement for IAD states, "*the use of tracheal wash cytology is insufficient for the diagnosis of IAD*" (7).

An increase in the relative number of tracheal neutrophils could be an adaptation to increased exercise and not necessarily a pathogenic response (84). McKane et al. (1993)(85) found that BALF from horses in active training or racing contained approximately twice as many neutrophils as those undertaking slow work. Similar results have been found in human athletes (84).

2.6.5 Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL), colloquially known as a 'lung wash', is a method for the recovery of respiratory secretions that line the distal airways and alveoli. The procedure is considered safe, repeatable and has few reported complications (74). Good correlation between BAL fluid (BALF) cytology and histopathological pulmonary cellular patterns has been reported (86). In general, tracheal wash and BALF cytology findings do not correlate well and the latter allows a better assessment of peripheral lung disease (87). Tracheal wash samples may not accurately reflect inflammatory activity in the bronchioles and alveoli and thus the BAL is considered a more representative sample (36).

There is no standardized equine BAL technique, with wide variations in both lavage volume and methodology used worldwide. The procedure can be performed blindly using a specialised commercial tube or under guidance with a

flexible (>2 m) endoscope (74,76,86,88). Because the unprotected tube lumen is likely to be in contact with resident bacteria found in the upper airways, BALF is used primarily for cytological examination and not for microbial culture (36).

The cell population of the equine lower airways is essentially uniform and a single BALF sample collected from any lung segment is considered to be representative of the entire lung (89). Pulmonary alveolar macrophages and lymphocytes are the predominant cell types found in BALF (86). Published reference values for BALF cell percentages in clinically healthy horses as defined by the Havemeyer Foundation Workshop in 2002 in clinically healthy horses are: 60% alveolar macrophages, 30% lymphocytes, <5% neutrophils, <2% mast cells and, <1% eosinophils (36). Epithelial cells are rare, although a few non-ciliated bronchial epithelial or goblet cells may be observed (See Table 1).

Published reference values for total cell counts of equine BALF vary, possibly due to differences in BAL volume and techniques used. Total cell counts (TCC) are deemed of little diagnostic use due to the variable and unknown dilution factor of BALF (90). Another potential source of error in TCC is the haemocytometer count, which is reportedly a relatively inaccurate measurement (91).

Table 1: Normal BALF cytological findings in horses

(*: data expressed as median value)

Number of Animals	Breed	Age	Macrophage (%) $\bar{x}$ ± SD	Lymphocytes (%) $\bar{x}$ ± SD	Neutrophils (%) $\bar{x}$ ± SD	Mast Cells (%) $\bar{x}$ ± SD	Eosinophils (%) $\bar{x}$ ± SD	Airway reactivity PC35 (mg/mL) $\bar{x}$ ± SD	Author
86	TB	4-6	56.0 ± 1.4	36.7 ± 1.6	5.9 ± 0.5	0.8 ± 0.2	0.5 ± 0.1		Clark et al., 1995
15	XB + TB	1-19	72 ± 10	18 ± 3	5 ± 4	1 ± 1	2 ± 4		Fogarty, 1990
6	SB	4-8	43.0 ± 4.5	50.5 ± 4.9	2.0 ± 1.4	3.0 ± 1.8	0.5 ± 0.9		Tee et al., 2012
6	SB	3.5 ± 1.0	67.7*	31.5*	0.4*	1.0*	0.3*	21.4	Hare and Viel, 1998
15	Mix	2-11	53.5 ± 9.6	43.9 ± 10.9	3.79 ± 2.0	2.67 ± 1.3	0.18 ± 0.5	16.1 ± 10.5	Pacheco et al., 2014
16	Mix	> 20	40.6 ± 11.3	53.4 ± 9.4	6.1 ± 4.8	1.92 ± 1.5	0.18 ± 0.4	18.0 ± 11.1	Pacheco et al., 2014
11	TB	2-6	65 ± 6.2	28 ± 5.8	7 ± 3.3	0.2 ± 0.6	0		Fogarty and Buckley, 1991
13	WB	3-7	45.9 ± 11	34.9 ± 7.1	9.1 ± 7.1	4.5 ± 2.6	2.1 ± 7.3		Gerber et al., 2003
13	WB	13-19	49.7 ± 9.6	32.9 ± 9.5	9.1 ± 8.9	5.5 ± 2.8	0.5 ± 0.7		Gerber et al., 2003
9	Not stated	8.3 ± 4.3	57.1± 10.3	31.4 ± 13.0	6.8 ± 2.7	0.3 ± 0.5	1.5 ± 0.8		Couëtil et al., 2001

2.6.5.1 BAL volume

The most important technical factor that influences reported 'normal' valves for BALF differential cell count amongst various research groups is differences in the volume of fluid used for lavage. This appears to be of critical importance when comparing studies. Depending on the technique used neutrophils constitute less than 10% of total number of nucleated cells (80,86). This upper limit of normal reduces to 5% when larger fluid volumes are used (7). Human BALF studies have shown that lavage volumes less than 20 mL increase the proportion of bronchial epithelial cells and neutrophils and decrease the proportion of alveolar macrophages and lymphocytes compared to larger volume lavages (92). Hence, the first aliquot is most representative of bronchial material, whereas subsequent aliquots contain more bronchoalveolar secretions (93). If smaller lavage volumes are used, the volume recovered is lower and the cell differential richer in neutrophils and contains fewer mast cells (80).

Controversy also exists on the necessity to analyse one or several BAL aliquots for the diagnosis of lung diseases. One study refutes the need for higher volumes, showing that sequential aliquots of saline (100 mL each) did not significantly differ from a pooled sample (300 mL)(94), but mast cell percentages have been shown to be higher in the first aliquot (95). Pickles et al. (2002) reported that although variations were observed, there was no significant difference in nucleated or differential cell counts among sequential and pooled BALF aliquots in clinically normal horses and horses with RAO (95). The author does however

acknowledge that lack of statistical significance may be due to the small sample size in the study (n=21).

Pooling aliquots or using different lavage volumes has been documented to influence the number of mast cells recovered and identified in BALF (80,96). Jean et al. (2011) found a significantly higher percentage of neutrophils and lower percentage of macrophages in first aliquot compared to second aliquot for horses in both control and RAO groups (97). The second aliquot diffuses farther into the airways to reach the alveolar spaces.

The use of higher volume lavage (300-500 mL) overcomes these reported inconsistencies, most likely because of the greater surface area sampled (98). An international workshop on equine chronic airway disease recommended the use of between 300 and 500 ml of lavage fluid to obtain consistent results comparable to the published reference ranges (2). Pleural leakage of fluid has been shown to occur with continuous infusion of volumes in excess of 450 ml (86).

A variety of inflammatory cell profiles may be displayed in horses with IAD (36, 74). One or more inflammatory cell types may be elevated as follows: mastocytosis (mast cells >2%), eosinophilia (eosinophils >1%)(99), or mildly to moderately increased neutrophils (16,19,50,73,91,100). Sanchez et al. (2005) reported that the absolute number of lymphocytes and the relative percentage of lymphocytes were significantly higher in horses with IAD than other animals (18). It has been suggested that these different inflammatory cell profiles may

reflect different aetiologies or environmental exposures and immune responses to disease or alternatively may represent different stages of disease (36).

Although some authors have reported an association between mast cells in excess of 2% in BALF and exercise intolerance in racehorses (64,101,102), others question the significance of this and report ranges of 0.7 to 12.3% for mast cells in BALF from horses showing no clinical evidence of respiratory tract disease (90).

Table 2: BAL cytology of horses with clinical signs of IAD - poor performance or coughing plus increased tracheal mucus

(*: data expressed as median value)

Number of Animals	Breed	Age	Macrophage (%) $\overline{x}$ ± SD	Lymphocytes (%) $\overline{x}$ ± SD	Neutrophils (%) $\overline{x}$ ± SD	Mast Cells (%) $\overline{x}$ ± SD	Eosinophils (%) $\overline{x}$ ± SD	Airway reactivity PC35 (mg/mL) $\overline{x}$ ± SD	Author
5	SB	2.6 ± 0.9	58.6*	25.8*	0.8*	1.4*	11.8*		Hare and Viel, 1998
65	TB	2-9	64 ± 15.2	23 ± 11.4	13 ± 12	0.3 ± 0.7	0.1 ± 0.3		Fogarty and Buckley, 1991
62	TB	1-7	59.0 ± 9.7	31.3 ± 9.3	8.8 ± 6.4		0.5 ± 3.1		McKane et al., 1993
20	Not stated	2-24	45.6 ± 2.0	48.5 ± 1.7	2.8 ± 0.3	2.7± 0.4	0.17 ± 0.05	5.88 ± 1.06	Hoffman et al., 1998
5	Not stated	16.6 ± 9.0	42.0 ± 17.6	36.3 ± 6.8	20.4 ± 9.3	1.5 ± 1.3	0.5 ± 1.0		Couëtil et al., 2001

2.6.5.2 BAL Catheter Location

A blindly passed BAL catheter has been shown to sample the dorso-caudal area of the lung (86). It is assumed that a single BALF sample taken from any site is representative of the entire lung and the technique is therefore recommended for the diagnosis of diffuse rather than localised diseases of the lung (86). Lung disease localised to other areas of the lung would not be detected, although it may however result in an impairment of pulmonary function and abnormal clinical signs. Conversely, if the cytological changes observed in the BALF are abnormal and are localized to the caudal areas of the lung, function of the entire lung may not be measurably decreased (17,37). Some caution must therefore be used in assessing the functional significance of abnormal BAL findings.

In a study of horses presented with poor performance, Davidson et al. (2011) found that 41% of horses with abnormal BAL cytology had impaired blood gas exchange but they could not identify a significant relationship between IAD and increased exercise-induced hypoxemia (37). This supports the hypothesis that the BAL sample may not be representative of the global lung pathology.

The presence of inflammatory cells in BALF is dependent on an intraepithelial or intraluminal position in the small airways. In a study by Fogarty (1990), pulmonary eosinophils were only detected in BALF in 40% of lungs that had eosinophilic infiltrates on histopathology. This finding is mainly due to the interstitial position of the eosinophil and highlights that interstitial pulmonary disease will not be reflected to the same degree of accuracy as bronchoalveolar

disease (86). Likewise, mast cell detection in BALF is dependent on an intraepithelial or intraluminal position of these cells in the small airways (86).

Although it is assumed that cell populations are similar in BALF recovered from different sites of the lung, significant differences in the relative percentage of mast cells have been reported between the left and right lungs (80,97). There is no clear explanation for these reported site differences in the relative percentage of mast cells.

2.6.5.3 Effect of prior BAL on subsequent cytology

Studies have shown that the BAL procedure has no effect on subsequent BAL cytology (103,104) and does not result in histopathological evidence of inflammation (86). Others have suggested however that the procedure may induce transient localised inflammation in the sampled region for at least 48 hours, as demonstrated by an increase in neutrophils (105). There is no evidence that BAL causes any significant disturbance in lung function; indeed, in one study, the BAL procedure was associated with an improvement in airway function, which the group attributed to the removal of mucus (106) by the wash.

2.6.5.4 Effect of exercise on BALF cytology

The effect of exercise on the cytological evaluation of respiratory fluids remains controversial. It is customary to perform BAL after exercise rather than before, because mucus can be observed more readily at this time (98). It is suggested

that higher intensity exercise may be associated with a low-grade inflammatory response in the lower airway due to increased exposure to aerosolized irritants and other environmental factors during maximum ventilation (85). This theory is supported by a number of studies in which increased BALF neutrophil counts have been found in horses in higher intensity training (19,37,57,85). Low volumes of lavage fluid were used in both McKane and co-worker's (1993) and Courouce-Malblanc and co-worker's (2010) studies, which is likely to have had an influence on their results. Although Couetil and Denicola (1999) demonstrated higher neutrophil numbers in post exercise BAL samples, this increase was small and not significant (19). A study by Clark et al. (1995) concluded that exercise did not alter BAL cytology (103) suggesting that training does not elicit a significant inflammatory response in the lower airway.

2.6.5.5 Laboratory analysis

The BALF recovered is pooled and a well-mixed sample is placed into sterile containers containing EDTA for centrifugation (600g, 5 min). It is important to centrifuge the samples to produce a uniform, monolayer and adequate number of cells to examine microscopically (88). The European Society of Pneumonology Task Group on BAL recommends a cytocentrifugation speed of 800 rpm (90 g) for optimal lymphocyte preservation in human BALF (107).

A smear slide may also be prepared. Pickles et al. (96) showed that smear preparations are reliable for the cytological diagnosis of equine neutrophilic pulmonary disease. The study found no significant difference in neutrophil

differential cell count between cytocentrifuged and smear preparations of BALF. Smear preparations did produce smaller, darker staining cells, making cytological identification more difficult than on cytocentrifuged preparations. Smears should be air-dried as quickly as possible to preserve cell quality (88).

In a study to evaluate the effects of time, temperature and different fixatives on BALF cytology, Pickles et al. (2002) found that storage at 4°C was optimal for diagnostic purposes (108). The research group found that BALF can generally be stored at room temperature if processed within 8 h, but should be refrigerated if processing is to take place 8-24 h after collection, as cell viability decreases with increasing temperature and time. Unfixed samples showed a progressive deterioration in cell morphology with increasing time. Therefore, it is recommended that if BALF is to be processed over 24 hours after collection, a formalin- or alcohol-based fixative should be added (108).

Cytocentrifuge or smear slides can be stained with a variety of commercial stains, including Romanowsky (Diff-Quik®), Wright's-Giemsa, May-Grunwald-Giemsa, and Leishman's stain. Prussian blue stain may be used for staining of hemosiderin if desired. The Romanowsky stain is the easiest to use in the practice environment but has the disadvantage that mast cell granules stain poorly as the stain is not metachromatic (109), making the enumeration of mast cells dependent on cell morphology (110). A number of studies on lower respiratory tract cytology in horses have only used the Romanowsky stain and in most of these low numbers of mast cells have been identified (85), potentially contributing a bias to the reported results.

Toluidine Blue has been shown to have the greatest sensitivity for the detection of mast cells (110), but this stain does not allow identification of other cell types. A general recommendation is to use the Romanowsky stain for differential cell counts and Toluidine Blue (30 minutes) for mast cell differential counts, which are counted separately (98).

The 400-cell leukocyte differential count, using a bright-field microscope at high power (630 to 1000 x) magnification, is a standard cell counting method for equine BAL samples (98). A differential count of particular classes of inflammatory cells is expressed as a percentage of the total inflammatory cells rather than enumeration of absolute numbers per unit volume of sample (94). This method does not take into account cell density and uneven cell distribution in a cytocentrifuged preparation. A recent study evaluating the inter-rater reliability of 400-cell leucocyte differential counts in equine BAL compared with an alternative method in which 5 microscopic fields were evaluated at 500 x magnification found that although reliability was higher for the 5 field method, overall the difference between methods was not significant (111).

2.6.6 Pulmonary function testing

Pulmonary function testing (PFT) can aid in establishing a definitive diagnosis of IAD. The technique has the additional benefit of providing an objective method of monitoring a response to therapy. A variety of pulmonary function testing methods are available but few are non-invasive. The equipment is often

cumbersome, expensive or complicated and as a consequence use is generally limited to research facilities. Techniques used in horses include: 1) conventional lung mechanics using pleural pressure measurements; 2) forced oscillatory mechanics; 3) forced expiratory manoeuvres; and 4) plethysmography.

2.6.6.1 Conventional lung mechanics using pleural pressure measurements

The conventional lung mechanics technique is based on the oesophageal balloon technique described by Derksen and Robinson (112), and measures pressure and flow variations resulting from the horse's spontaneous breathing. Oesophageal pressure is measured by means of a balloon-tipped catheter advanced into the distal third of the thoracic oesophagus and connected to a pressure transducer. The other side of the pressure transducer is connected to the mask via a similar catheter. Transpulmonary pressure is then calculated from the difference between mask pressure and oesophageal pressure (76,113). Variables that may be measured include maximum change in transpulmonary pressure, tidal volume, inspiratory time, expiratory time, peak inspiratory flow rate, peak expiratory flow rate, breathing frequency and minute ventilation. Values for pulmonary resistance and dynamic lung compliance are then computed (114). The conventional lung mechanics technique using pleural pressure measurements in horses lacks sensitivity, and often only detects mechanical dysfunction when clinical signs of disease are apparent.

2.6.6.2 Forced oscillation techniques (oscillometry)

Forced oscillation techniques (FOT) measure the response of the respiratory system to external forces (115). Techniques are non-invasive, readily tolerated and require only a facemask on the patient. FOT impose sinusoidal oscillations from an external source of energy through the respiratory system to generate similar pressure flow responses from the respiratory system that are measured at the airway opening. Natural fluctuations of airway pressure and flow due to breathing are filtered out. The amplitude and synchrony of the flow and pressure oscillations from the respiratory system determine lung function by measuring total respiratory impedance in a specific spectrum of frequencies. Total impedance across the respiratory tract, is related to the behaviour of 3 independent forces that act in series: resistance, elastance, and inertance. Resistance describes pressure due to friction, elastance describes pressure due to tissue recoil, and inertance describes pressure related to acceleration of flow. As one factor changes relative to the other, total impedence varies (115). The impulse oscillometry system (IOS) is a non-invasive respiratory function test based on the forced oscillation principle and has been validated in horses (116). The system applies multi-frequency external signals, generated with a loudspeaker, and the respiratory system produces a pressure flow signal response measured by a pneumotachograph and pressure transducers. Total respiratory resistance and reactance is recorded (117). The IOS was more sensitive than the conventional lung mechanics method, based on the oesophageal balloon technique, in detecting changes in respiratory impedance in RAO-affected horses (118). Changes in airway resistance and reactance during

each phase of respiration were demonstrated in a population of young racehorses with apparent IAD (119).

2.6.6.3 Forced expiratory manoeuvres

Forced expiration (FE) is one of the most useful and commonly used pulmonary function tests for the early detection of small airway disease in humans (115,120). The FE technique requires patients to inhale until they have achieved total lung capacity and to then exhale as hard and completely as possible while expiratory flow and volume are recorded. Such manoeuvres can be performed in animals, but require the use of general anaesthesia to avoid interference of conscious respiratory movements with emptying of the lungs. FE manoeuvres are rarely performed in horses because the methods are invasive and cumbersome.

Couetil et al (2000) developed a minimally invasive and non-plethysmographic FE method for use in horses that were sedated but not anaesthetised. They concluded that peripheral airway obstruction was detectable through the analysis of the forced expiratory flow-volume (FEFV) curves (120). Horses with a history of inflammatory airway disease have lower values for forced expiratory flows compared to normal horses (114).

2.6.6.4 Plethysmography

Whole body plethysmography has been successfully used for respiratory measurements in human subjects since the mid-1950s. At an international

symposium on lung function and respiratory diseases in the horse in 1985 Beadle described his experiences with whole body plethysmography in horses with RAO (121). The plethysmograph described was a set of stocks welded to a square steel tubing chassis, which in turn was incorporated by an airtight box (Figure 1). The wall and ceiling of the box were made from 19 mm thick plywood attached to an angle iron framework and the floor was made from 6 mm thick steel plating. The door and window were sealed when closed by means of specially designed gaskets constructed from closed cell foam rubber weather-stripping material coated with latex rubber. Two breathing circuits were built into the box. Circuit 1 measured thoracic gas volume and circuit 2 measured alveolar pressures.

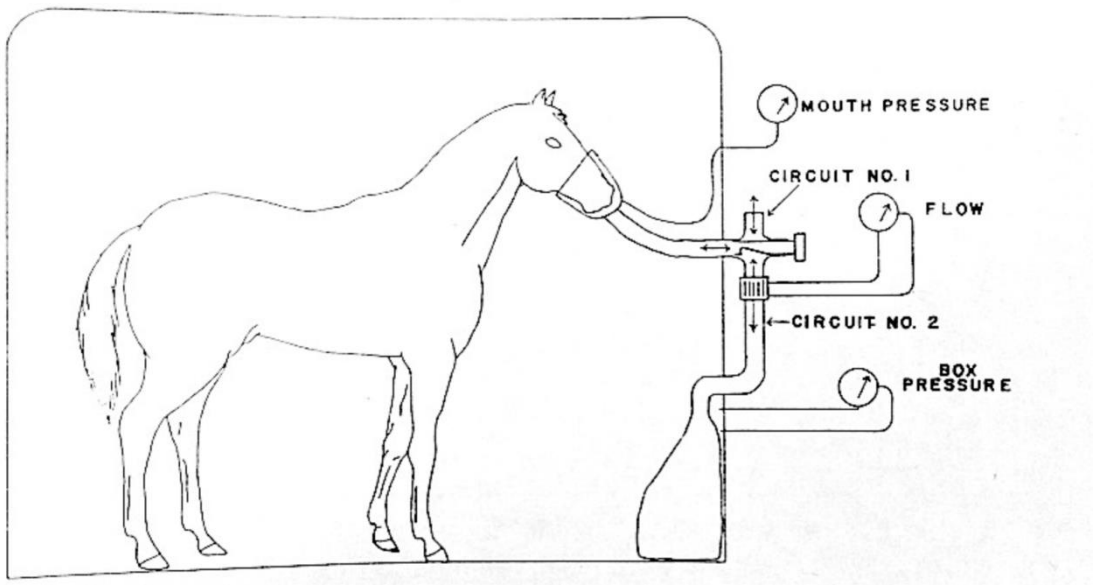

Figure 1: Box plethysmograph as described by Beadle (121)

Box-less or 'flowmetric' plethysmography (FP) is a recently developed non-invasive lung function testing method. In horses, FP (trade named "Open Pleth")

is conducted using a combination of external sensors placed on the body surface (respiratory inductance plethysmographic (RIP) bands) and a measure of flow at the nares by a pneumotachograph (Figure 2). Gas compression from airway obstruction results in discordance between the two sensors. The contribution of thoracic and diaphragmatic movements to the breathing pattern and thus thoraco-abdominal asynchrony can also be analysed (115). The sensitivity of the FP system is similar to conventional lung mechanic testing (115) and the test is coupled with bronchoprovocation methods to improve sensitivity.

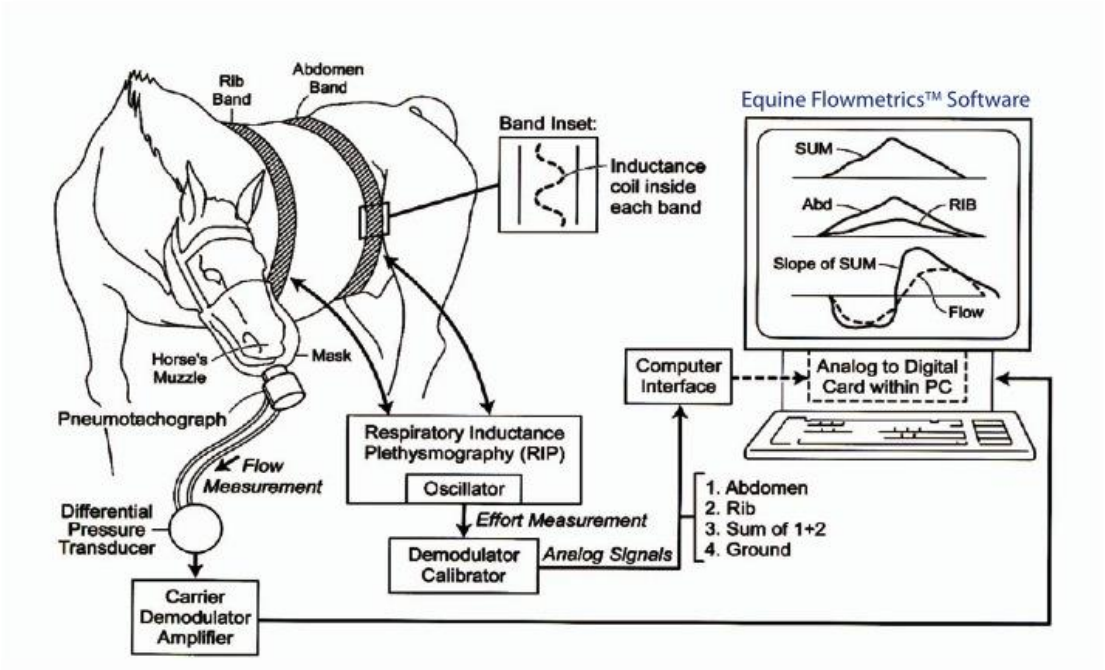

Figure 2: Schematic showing the components of the flowmetric plethysmographic method (122)

The respiratory ultrasonographic plethysmograph (RUP) has recently been validated in the horse (123). This system measures the stretching of compliant liquid-filled rubber tubes that are fastened around the thorax and abdomen. The

elastic tubes function as ultrasonic waveguides between an ultrasonic transmitter and receiver at the respective ends. An advantage of the RUP device compared to the RIP device is its relative resistance to the influence of electromagnetic interference (123).

2.6.6.5 Bronchoprovocation testing

Bronchial hyper-reactivity to pharmacological and physical stimuli is a characteristic of human asthma. The sensitivity of the airways to react with a bronchospasm, known as airway reactivity, can be determined in horses in a similar way to that in humans. Horses with an exaggerated response to pharmacological stimuli, such as a histamine aerosol challenge, are termed 'hyper-reactive' and airway hyper-reactivity (AHR) is thought to be a good marker for inflammation of the small airways (117).

The methods for bronchoprovocation in horses are adapted from those used in humans. The histamine bronchoprovocation test is typically performed to detect nonspecific AHR. Bronchial hyper-reactivity to intravenous administration of histamine was first described in horses with chronic airway disease in 1948 (124). In humans, histamine inhalation has been used as a test to provoke bronchoconstriction to determine the level of nonspecific airway reactivity in asthmatics since the mid-1940s. The test is based on Poiseuille's law, which states that resistance is inversely proportional to the radius of the airway lumen to the fourth power (20). Therefore even a slight decrease in radius with

exposure to a bronchoconstrictor agent during bronchoprovocation tests manifests an exaggerated change in airway resistance.

The histamine inhalation provocation test (HIPT) can also be carried out in horses and has been used as a method to identify nonspecific airway reactivity in horses since the mid-1980s (125). Early studies have demonstrated the use of HIPT in association with the conventional lung function testing method described above (section 2.6.6.1). After determination of baseline lung function values, the first test solution nebulised was the solvent for the histamine solutions. After the 2-minute test solution inhalation, the nebuliser adapter was exchanged for the flow-transducer, and lung function was tested about 60 s after inhalation. The following test solutions always contained histamine dihydrochloride with doubling concentrations. After inhalation of increasing concentrations of histamine solutions, horses demonstrate a decrease in dynamic compliance and an increase in airway resistance, work of breathing, and maximum intra-thoracic differences in pressure. A linear regression with the baseline value, the solvent value, and all values after inhalations of histamine solutions is carried out and the histamine concentration that resulted in a 35% reduction in dynamic compliance (C_{dyn}) is calculated. This concentration was called 35% provoking concentration (PC_{35} C_{dyn}). Nonspecific airway hyper-reactivity was not present in horses that did not have clinical signs of disease but present in 25% of horses with low grade lung disease and in all horses with severe lung disease (125). These findings suggested that horses with lung disease are hyper-reactive to inhaled histamine, the PC_{35} reflecting the degree of disease.

Klein (126) classified airway reactivity of horses in 1984. Normal reactivity is classified as a $PC_{35}C_{dyn}$ greater than 8 mg/mL histamine dihydrochloride (Table 3).

PC_{35}	Reactivity grade
> 8mg histamine/mL	Normal reactivity
Between 1 and 8 mg histamine/mL	Low grade hyperreactivity
Between 0.125 and 1 mg histamine/mL	Moderate hyperreactivity
<0.125 mg histamine/mL	High grade hyperreactivity

Table 3: Classification of the reactivity grades by *Klein* (1984)(126)

Histamine bronchoprovocation can be used in combination with all of the previously described pulmonary function tests to increase test sensitivity. In a prospective study investigating the reproducibility of airway responsiveness in horses using flowmetric plethysmography and histamine bronchoprovocation, Nolen-Walston et al showed acceptable reproducibility over time periods up to a year (127).

2.6.7 Association between BAL and PFT

Studies reporting the association between BAL cytology and PFT are limited. Couëtil and co-workers reported that horses with inflammatory airway disease, as diagnosed by medical history and clinical signs of cough and increased

tracheobronchial mucus, had significantly lower values for forced expiratory flows when compared to a group of normal horses (114). Higher relative BALF neutrophil percentages were found in the IAD group, but a significant difference was not found when compared to normal horses in their study. This was likely due to low sample size (114).

Significant correlations between BALF mast cell percentage and AHR (64, 101) and the relative eosinophil percentage and AHR (99) are reported. Impulse oscillometry system methods without histamine bronchoprovocation or respiratory challenge have also reported a correlation between elevated BALF eosinophils and mast cells with respiratory dysfunction (119).

Disjunction between BALF cytology and airway hyperreactivity has been reported, with some horses showing abnormal lung function in the face of normal BALF cytology, and vice versa (128). These studies are very limited and it was our primary aim of this study to further investigate and characterise the association between BALF cytology and pulmonary function testing with histamine bronchoprovocation methods.

CHAPTER 3. MATERIALS AND METHODS

Study design – The study consisted of two parts:

Part 1: Reliability testing

Pulmonary function testing and histamine bronchoprovocation was performed on horses on three separate occasions at one-week intervals. All lung function testing was performed in an air-conditioned, temperature-controlled environment.

Part 2: Data collection

Pulmonary function testing, histamine bronchoprovocation and a bronchoalveolar lavage were performed on horses as outlined below.

Animals - All animal testing was performed with the approval of Murdoch University Animal Ethics Committee (Permit number R2405/11).

- Part 1: In part 1 of the study, 10 adult horses from a university owned herd were included. Subjects consisted of 4 Thoroughbreds, 5 Standardbreds and one Arabian of which 8 were geldings and 2 were mares, aged from 5 to 20 years.
- Part 2: Thirty-eight healthy adult horses from a university owned herd were used for the study. Subjects consisted of 18 Thoroughbreds, 19 Standardbreds and one Arabian, aged from 4 to 27 years. Of these, 14 were geldings and 24 were mares.

None of the horses had a recent history or clinical signs of respiratory tract disease. Routine physical examination was within normal limits on all horses. All horses were housed in a large pasture for a minimum of 4 months before and throughout the study. Management remained consistent throughout the study.

Pulmonary function testing (PFT) - Pulmonary function testing and histamine bronchoprovocation were performed with a commercial flowmetric plethysmography system (Open Pleth™ using Equine Flowmetrics™ software). Prior to use, the system was calibrated as per manufacturer's instructions (Ambulatory Monitoring, Inc.). Each horse was restrained in metal stocks, sedated with detomidine (0.01 mg/kg), fitted with an equine mask, pneumotachograph (Figure 3), and abdominal and thoracic respiratory inductance plethysmography (RIP) bands (Figure 4). The thoracic RIP band was placed between the ninth and eleventh intercostal space and the abdominal band was placed immediately behind the last rib. Head position was maintained on a stand in a consistent horizontal position (Figure 5). Firstly, baseline pulmonary function measurements were recorded for 3 minutes. The computer software (Figure 6) calculated total airway impedance, by subtracting thoracic volume change from pneumotachograph flow at peak expiration (delta flow). Saline (0.9%) was then nebulised as a control for 2 minutes and again measurements were recorded for 3 minutes. A series of increasing doses of histamine diphosphate solution was then nebulised, starting with 4 mg/mL (4, 8, 16, 32 mg/mL). The histamine solution was nebulised for 2 minutes and lung function data was collected for 3 minutes. The test was discontinued when the measured

response exceeded baseline delta flow by 50% or a maximum of 32 mg/mL of histamine diphosphate was reached. Horses thus acted as their own control.

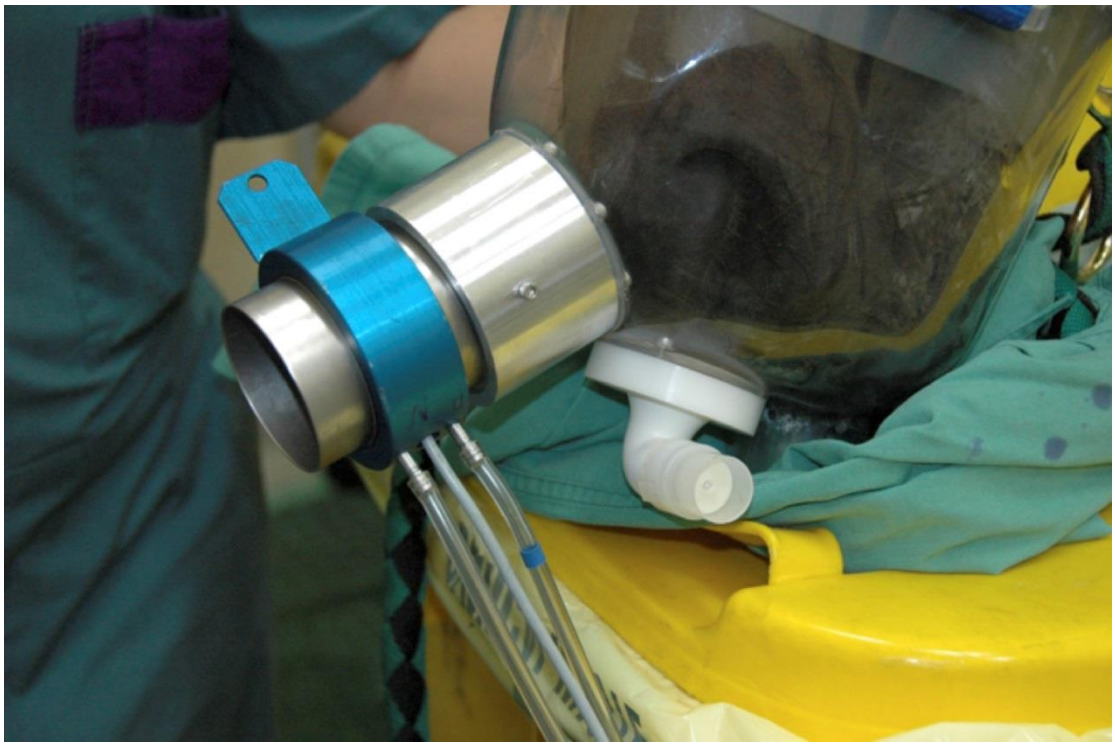

Figure 3: The horse is fitted with a mask and pneumotachograph.

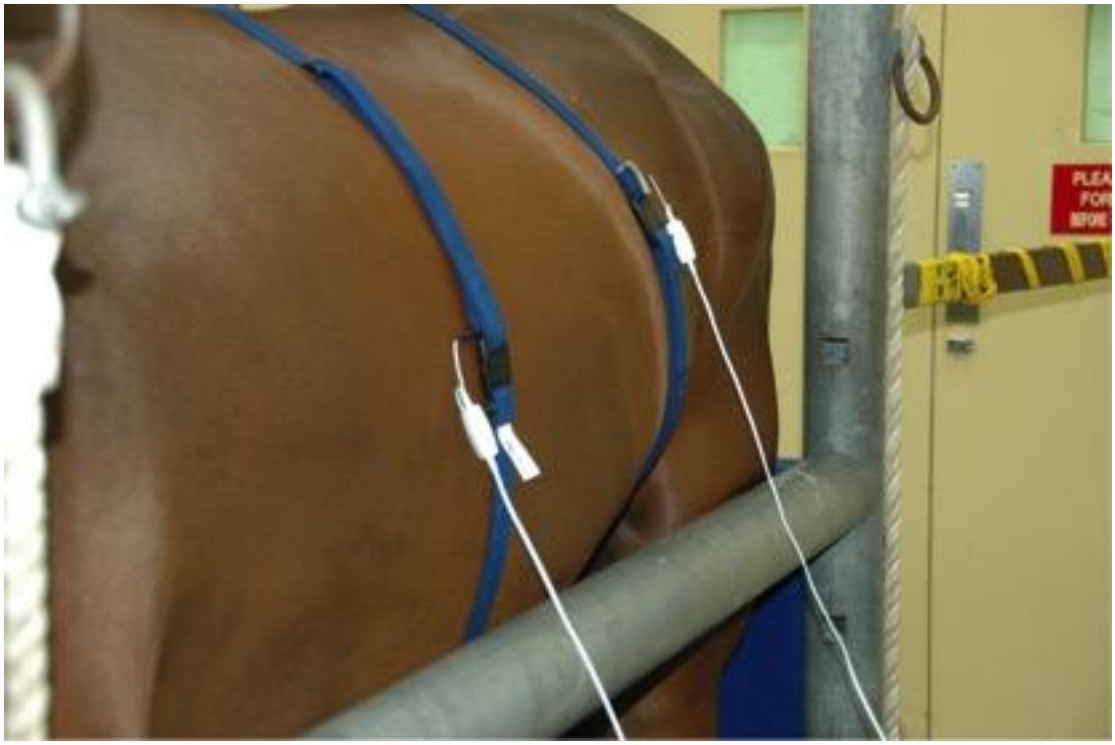

Figure 4: Thoracic and abdominal respiratory inductance plethysmography (RIP) bands were placed between the 9th and 11th intercostal spaces and behind the last rib respectively

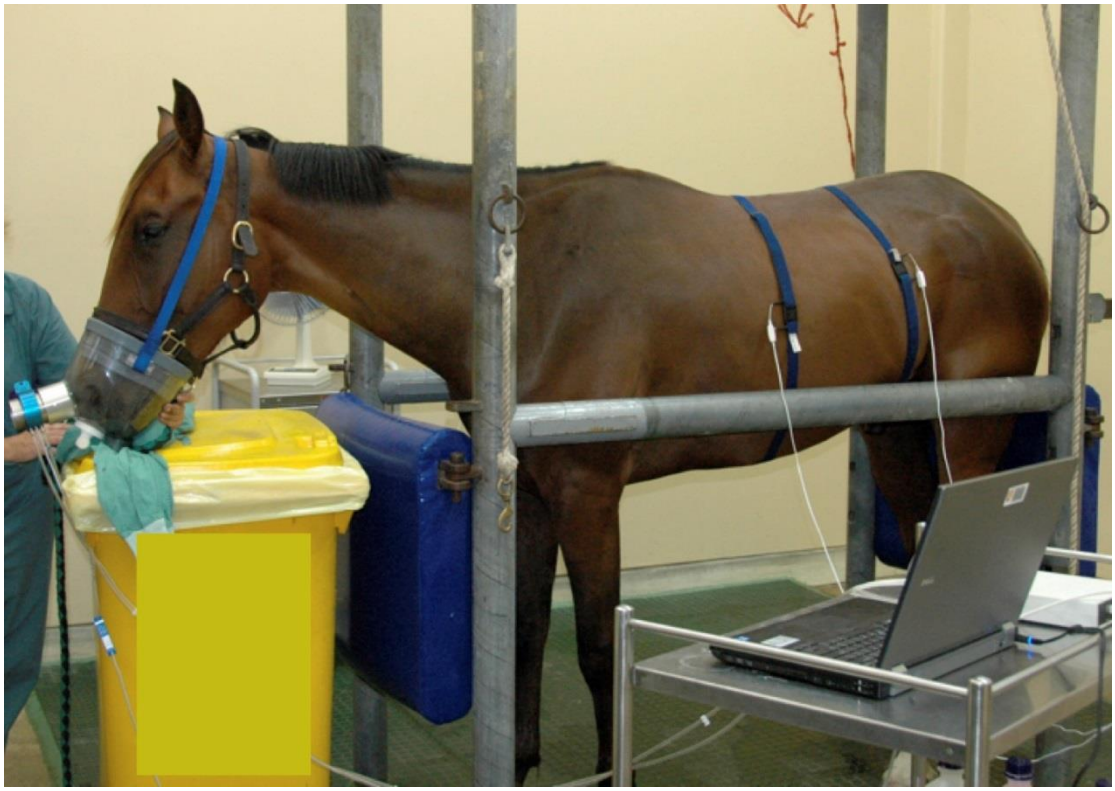

Figure 5: The horse's head is maintained in an upright position

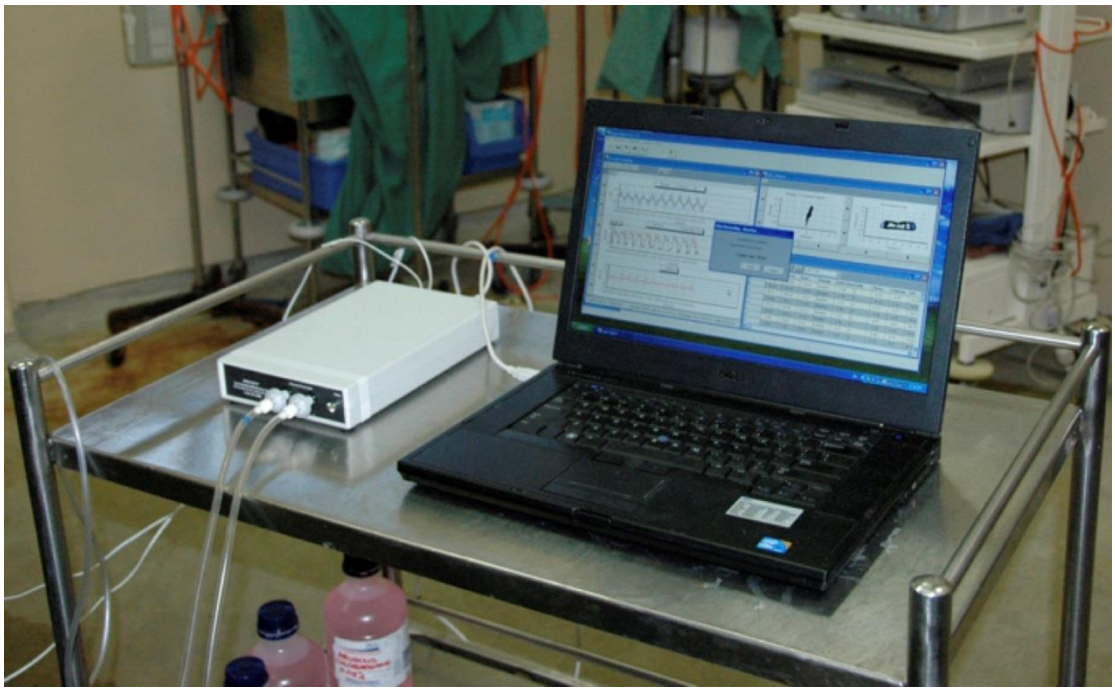

Figure 6: Computer system recording during the pulmonary function test

The data was manually reviewed and abnormal breaths due to excessive movement removed. A dose-response curve was then generated and the concentration of histamine that evoked a 35% increase in delta flow above baseline (PC_{35}) was computed by interpolation of the dose-response curve. Airway hyper-reactivity was expressed as a PC_{35} value. Airway hyper-reactivity was categorised into normal (PC_{35}: ≥ 8 mg/mL), mild (PC_{35}: 4-8 mg/mL), moderate (PC_{35}: 2-4 mg/mL) or severe (PC_{35}: < 2 mg/mL).

PC_{35} (mg/mL histamine)	Final airway reactivity category
> 8	Normal
4-8	Mild
2-4	Moderate
< 2	Severe

Table 4: Categories of airway reactivity

Horses were also categorised as being 'normal' if PC_{35} was greater than 8 mg/mL histamine or 'reactive' if PC_{35} was less than or equal to 8 mg/mL histamine.

PC_{35} (mg/mL histamine)	General airway reactivity category
> 8	Normal
≤ 8	'Reactive'

Table 5: General categories of airway reactivity

In part 1 of the study a subjective assessment of when the horse bronchoprovocated was also recorded. This involved documenting the concentration of histamine being nebulised when respiratory rate and depth increased 'significantly' above normal.

Bronchoalveolar lavage (BAL) – In part 2 of the study, a BAL was performed in all horses between 1 and 5 days post PFT. The majority of horses were tested at 24 hours post PFT. Horses were sedated with detomidine (0.01 mg/kg) and butorphanol tartrate (10-30 µg/kg) and were restrained in stocks with a twitch applied to the upper lip. BAL was performed using the blind technique (86, 88). A commercial cuffed BAL tube (Bivona ®) (Figure 7) with a diameter of 11 mm and a length of 244 cm was passed blindly through the ventral nasal meatus into the pharynx (Figure 8). The head was stretched into a horizontal position and the tube is advanced into the trachea, whereby a complete lack of resistance and manual rattling of the tube in the trachea confirmed its correct positioning. As the tube was passed down the trachea, 0.2% lidocaine (40 mL) was infused slowly through the tube (129) to desensitize the bronchial mucosa and reduce

coughing. The BAL tube was gently advanced until it could not be advanced further, the cuff on the BAL tube was then inflated with air (7-10 mL) and the tube gently pressed against the nasal septum with the thumb to secure its position. A total of 300 mL sterile isotonic saline at room temperature, divided into two aliquots of firstly 180 mL and then 120 mL, was infused into the alveolar space using 60 mL syringes (Figure 9). After each aliquot was infused it was gently aspirated (Figure 10) and once no further fluid was recovered on aspiration of the final infusion the cuff was deflated and the BAL tube removed. The 2 samples were subsequently pooled in a sterile container (Figure 9). BAL samples were considered adequate if they contained frothy material denoting the presence of surfactant (50) and there was greater than 50% of the volume retrieved. Samples were immediately placed on ice and transported within 30 minutes to the laboratory.

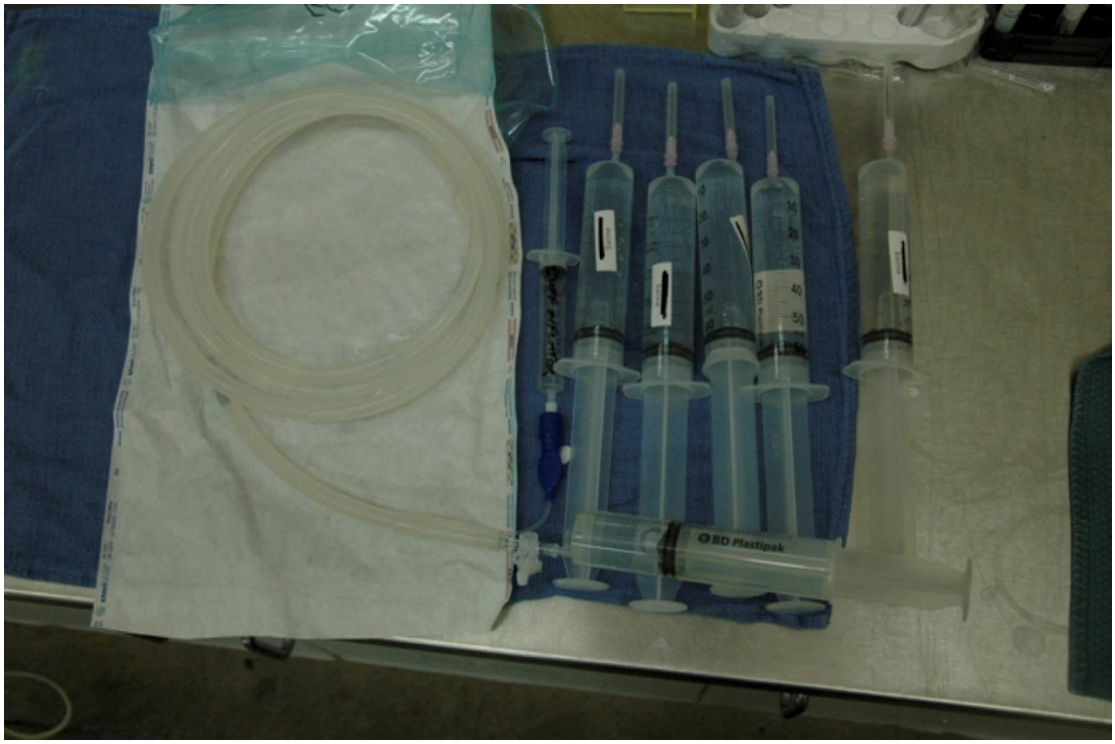

Figure 7: Bronchoalveolar lavage tube primed with 0.2% lidocaine with cuff inflator syringe attached and 5 x 60 mL syringes prefilled with 0.9% saline

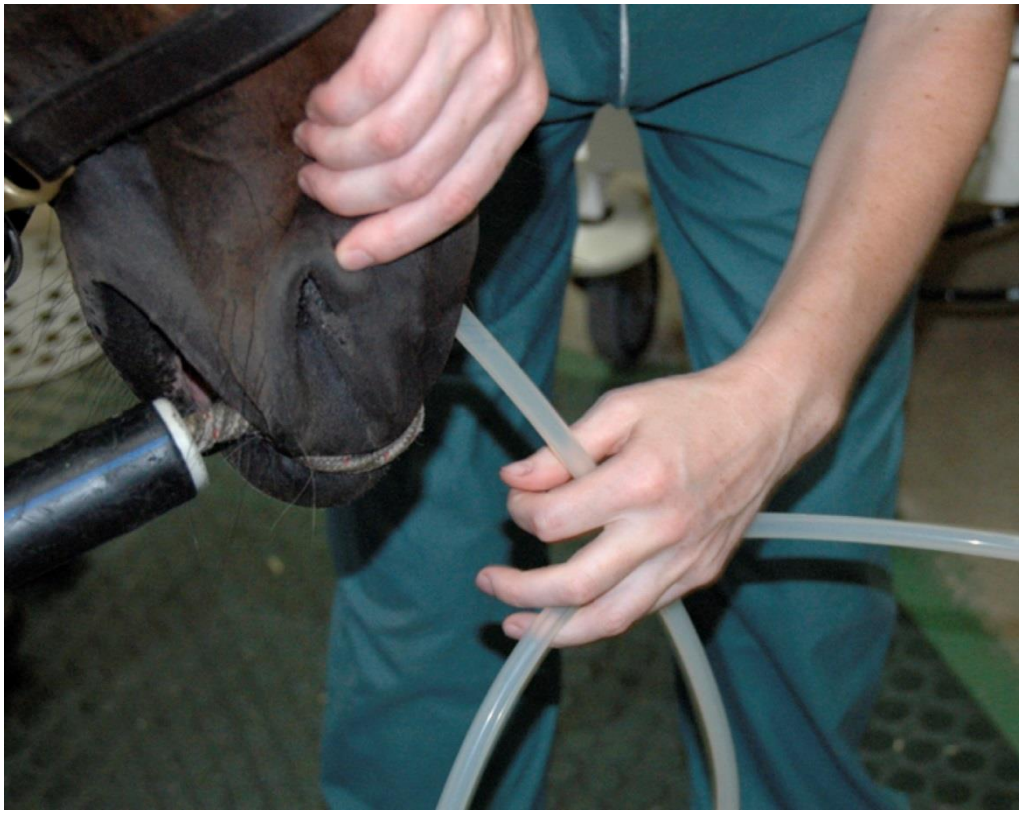

Figure 8: The horse is sedated and restrained in stocks with a nose twitch applied. The BAL tube is advanced up the nostril and into the trachea.

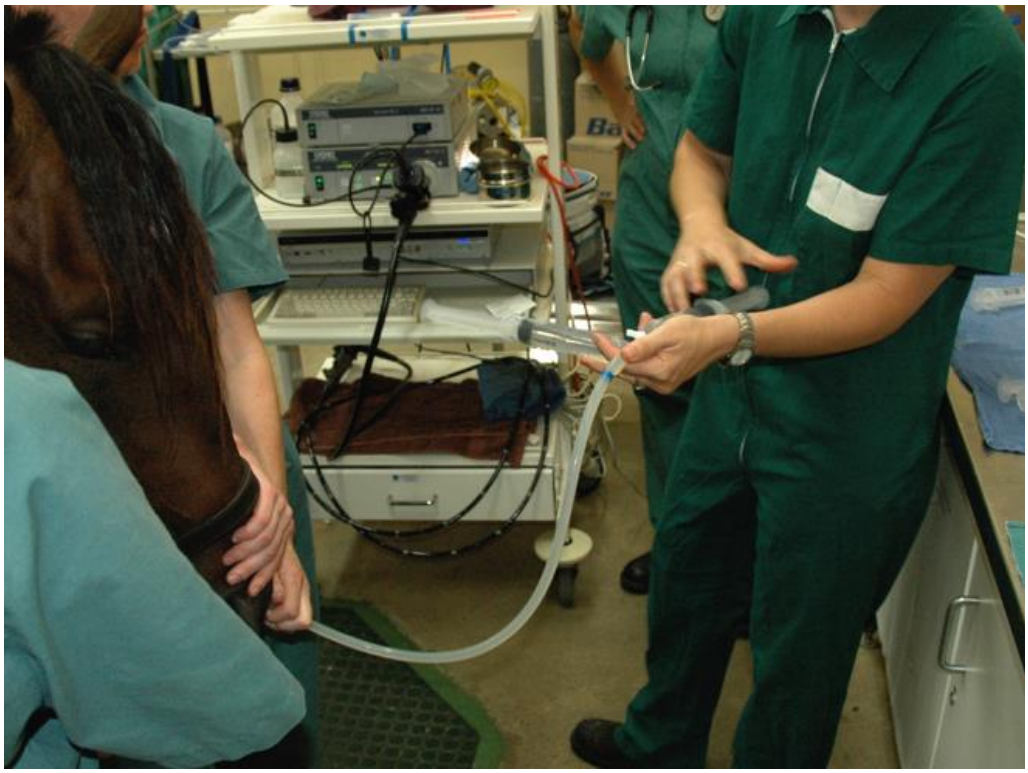

Figure 9: Bronchoalveolar lavage tube is gently pressed against the nasal septum with the thumb to secure its position at the nostril while saline is infused using 60 mL syringes

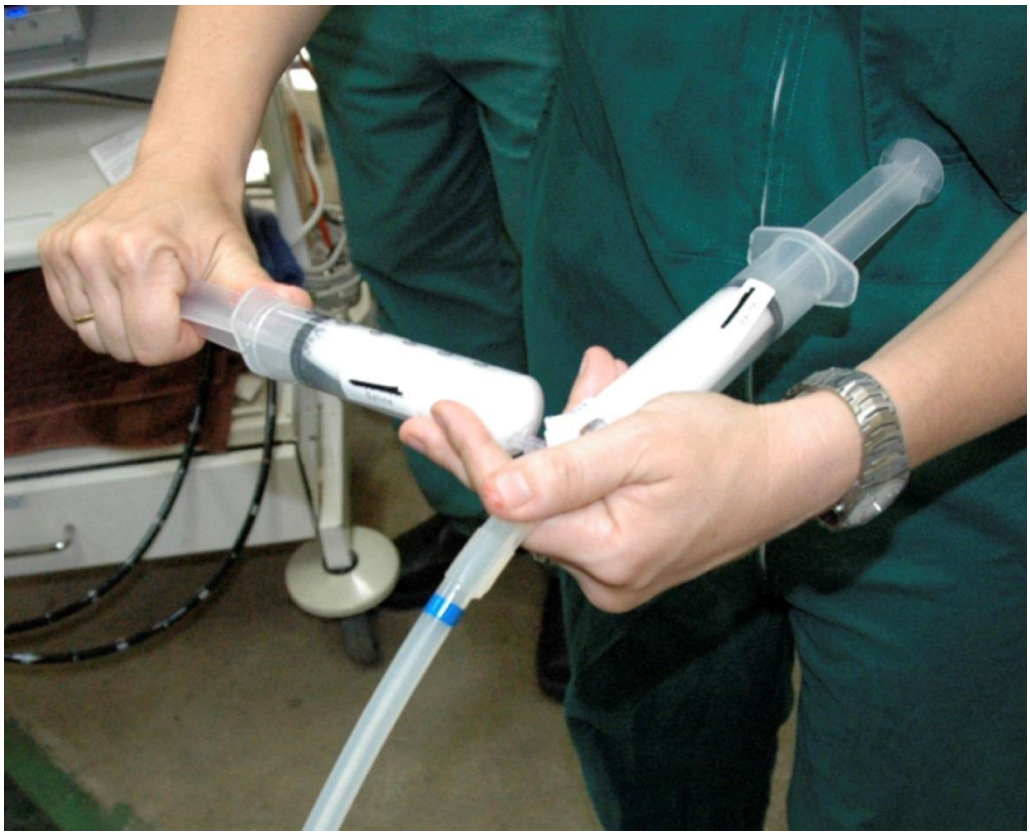

Figure 10: After each aliquot of saline is infused it is then gently aspirated using 60 mL syringes

Figure 11: Sterile pot containing the two-pooled BAL aliquots. The frothy material on top denotes the presence of surfactant.

Sample handling - All samples were processed within 30 minutes of collection. Twenty millilitres of BALF was centrifuged at 2500 *g* for 10 minutes, the supernatant was collected and frozen to -80 degree Celsius (this was used in another study). Another sample of BALF was stained with methylene blue dye and total nucleated cells were counted manually using a Neubauer haemocytometer. Slides were prepared by cytocentrifugation of the remaining pooled sample and stained with Leishman's stain. Differential cytological counts of all samples were performed by the same person, an experienced clinical pathologist, by counting a minimum of 400 cells using high power (630 x) light microscopy. Cells were classified as the percentage of macrophages, lymphocytes, neutrophils, mast cells and eosinophils.

Data analysis - In part 1, reliability was investigated using an intra-class correlation coefficient (Type C) for both objective and subjective data.

In part 2, data were organised into separate datasets based on inflammatory cell responses observed and categorized as follows:

0) Normal
1) Mastocytosis only: mast cells > 2%
2) Eosinophilic response only: eosinophils ≥ 1%
3) Neutrophilic response only: neutrophils >5%
4) Mixed type 1 response: neutrophils >5% plus mast cells >2%
5) Mixed type 2 response: mast cells > 2% plus eosinophils ≥1%
6) Mixed type 3 response: neutrophils >5% plus eosinophils ≥1%
7) Mixed type 4 response: neutrophils >5% plus mast cells > 2% plus eosinophils ≥1%

The data were not normally distributed on the basis of a significant Shapiro-Wilk statistic. Consequently a Spearman's correlation was used to assess age, total nucleated cell count and inflammatory cell responses observed and airway hyperreactivity (PC_{35}). Chi-squared analysis was used to compare all categories of inflammatory cell response (0-7) observed with the final category of airway reactivity (normal, mild, moderate or severe) and the general category of airway reactivity ('normal' or 'reactive') in every horse. Comparisons between categories using the independent variables relative cell percentage and PC_{35} values were undertaken using a Mann-Whitney U test. Significance was defined

as $P < 0.05$. All statistical analyses were performed by use of commercial software (IBM SPSS statistics, version 21).

CHAPTER 4. RESULTS

Part one

In part one of the study, 30 pulmonary function tests (PFT) with histamine bronchoprovocation were performed. On manual review of the PFT data, all 3 tests for horse number 10 and one test for horses' number one and 6 were deemed to be inaccurate, and these test results were excluded from data analysis. Two horses developed nasal oedema during pulmonary function testing on 2 individual occasions, precluding use of data in these instances. Objective data was collected in 23 instances and indices of airway reactivity (PC_{35} values) are listed in Table 6 below. Subjective data was collected in all thirty horses (Table 7).

Table 6: Objective data for part 1 of study showing PC_{35} values

Horse No.	PC_{35} (mg histamine/mL)		
	Week 1	Week 2	Week 3
1	*	10.77	4.69
2	2.14	5.51	5.44
3	9.27	8.25	NO
4	11.01	9.08	10.84
5	6.29	5.01	5.15
6	3.26	*	5.34
7	3.53	7.23	5.24
8	6.28	4.13	5.24
9	NO	12.64	5.44

Horse No.	PC_{35} (mg histamine/mL)		
	Week 1	Week 2	Week 3
10	*	*	*

*Result removed as deemed inaccurate after manual review; NO: Nasal oedema

A Type C intra-class correlation coefficient (ICC) was calculated for single measures for both objective and subjective data. The ICC for objective data was 0.655 (95% CI: 0.098, 0.952; significance: 0.011) and for subjective data was 0.932 (95% CI: 0.773, 0.987; significance: < 0.001).

Table 7: Subjective data for part 1 of study showing histamine concentration at which horses subjectively bronchoprovocated

Horse No.	Histamine concentration (mg/mL)		
	Week 1	Week 2	Week 3
1	8	8	8
2	8	8	8
3	16	16	NO
4	16	16	16
5	8	8	8
6	4	8	8
7	4	8	8
8	8	8	#
9	NO	16	8
10	16	16	16

- horse was clinically normal when test was stopped; NO: nasal oedema

Part 2

In part two of the study, 5 horses had incomplete data for airway reactivity due to failure of the pulmonary function-testing unit and were thus omitted from data analyses. All horses were clinically normal on routine physical examination. A BAL was performed on all horses. No adverse effects of the lavage procedure were documented. Cell differential percentages, category of inflammatory cell response, PC_{35} values and airway reactivity category are listed in Table 8 below (see Appendix 1 for table of raw numbers). Cell percentages have been rounded to average numbers and thus total percentages of cells do not always equate to 100%.

Table 8: BAL cell differential percentages and PC_{35} values

Horse No.	BAL cell differential percentages					Category of inflammatory cell response	PC_{35} (mg/mL)	Final category of airway reactivity
	Mac	Lymph	Neut	Mast	Eos			
1	52	38	5	4	0	1	1.63	Severe
2	48	38	12	1	0	3	2.26	Moderate
3	67	22	2.5	3	4	5	2.14	Moderate
4	31	32	5	1	30	2	4.69	Mild
5	70	20	8	0.5	0.5	3	4.83	Mild
6	50	31	14	4	1	7	NR	NR
7	46	45	7	1.5	0	3	5.34	Mild
8	72	12	10	2	2	6	5.15	Mild
9	52	21	12.5	4	10	7	4.13	Mild

Horse No.	BAL cell differential percentages					Category of inflammatory cell response	PC_{35} (mg/mL)	Final category of airway reactivity
	Mac	Lymph	Neut	Mast	Eos			
10	59	25	7	5	3	7	5.44	Mild
11	52	28	5	2.5	22	5	5.44	Mild
12	47	41	10	1.5	0	3	6.84	Mild
13	74	15	4	3	5	5	8.25	Normal
14	52	36	8	3	1	7	10.59	Normal
15	51	33	13	1.5	1	6	9.33	Normal
16	74	15	6	3	1	7	11.8	Normal
17	56	22	8	3	10	7	12.94	Normal
18	41	49	6	3	1	7	15.25	Normal
19	63	26	5	3	2	5	16.00	Normal
20	59	32	5	3	0	1	15.90	Normal
21	46	38	10	6	0	4	4.69	Mild
22	48	39	9	5	3	7	9.19	Normal
23	35	55	3	4	3	5	0.84	Severe
24	43	45	7	2	1	6	NR	NR
25	60	23	4	5	6	5	NR	NR
26	66	27	5	1	0	0	7.07	Mild
27	39	55	4	1	0	0	13.95	Normal
28	66	22	4	6	0	1	15.44	Normal
29	52	29	16	3	0	4	22.29	Normal

Horse No.	BAL cell differential percentages					Category of inflammatory cell response	PC_{35} (mg/mL)	Final category of airway reactivity
	Mac	Lymph	Neut	Mast	Eos			
30	63	22	10	2	2	6	18.60	Normal
31	76	10	9	5	0	4	5.14	Mild
32	39	40	13	7	0	4	31.93	Normal
33	39	38	20	3	0	4	3.30	Moderate
34	30	38	28	2	0	3	12.08	Normal
35	36	15	20	4	24	7	NR	NR
36	38	42	12	6	1	7	NR	NR
37	20	35	39	0	2	6	9.00	Normal
38	40	36	17	3	3	7	1.18	Severe

Mac: macrophages; lymph: lymphocytes; neut: neutrophils; mast: mast cells; and eos: eosinophils; abnormal cell percentages are highlighted in red; NR: not recorded

Bronchoalveolar lavage cytology results

Ninety-five per cent (36/38) of horses were diagnosed with inflammatory airway disease in respect to BAL cytological findings as per definition of the disease by the Havemeyer Foundation Workshop in 2002 (36), with the exception that eosinophil percentages greater than or equal to 1% were used in this study. Twenty-three horses (61%) were found to have a pulmonary eosinophilia and 3 horses had eosinophil percentages exceeding 20%.

Pulmonary function test results

Airway hyperreactivity (PC_{35} < 8 mg/mL) was demonstrated in 52% (17/33) of horses examined. Three horses were severely hyperreactive (PC_{35} < 2 mg/mL), 3 were moderately reactive (PC_{35} 2-4 mg/mL) and 11 were mildly reactive (PC_{35} 4-8 mg/mL).

Categories of inflammatory cell response

Table 9 below outlines the number of horses per category of inflammatory cell response. Twenty-seven horses (71%) were found to have a mixed inflammatory cell response, 5 (13%) had a sole neutrophilic response, 3 had a sole mastocytosis and one horse a sole eosinophilic response.

Table 9: Outline of number of horses and mean PC_{35} per inflammatory cell response category

Category of inflammatory cell response	Type of inflammatory cell response	Number of horses in category	Mean PC_{35} for category (mg/mL histamine ± SD)
0	Normal	2	10.51 ± 4.85
1	Mast cells only > 2%	3	10.99 ± 8.11

Category of inflammatory cell response	Type of inflammatory cell response	Number of horses in category	Mean PC_{35} for category (mg/mL histamine ± SD)
2	Eos only ≥ 1%	1	4.69
3	Neuts only > 5%	5	6.27 ± 3.64
4	Mixed type1 Neuts > 5% plus mast > 2%	5	13.47 ± 12.93
5	Mixed type 2 Mast > 2% plus eos ≥ 1%	6	6.53 ± 6.03
6	Mixed type 3 Neuts > 5% plus eos ≥ 1%	5	10.52 ± 5.71
7	Mixed type 4 Neuts > 5% plus mast > 2% plus eos ≥ 1%	11	8.82 ± 4.82

Mac: macrophages; lymph: lymphocytes; neuts: neutrophils; mast: mast cells; and eos: eosinophils

No correlation was found between age, total cell count or inflammatory cell responses and indices of airway reactivity. No correlation was found between category of inflammatory cell response observed and general or final category of airway reactivity.

Table 10 below outlines the mean neutrophil, mast cell and eosinophil percentages and general category of airway reactivity.

Table 10: Mean independent variable relative cell percentages and general category of airway reactivity

PC_{35} (mg/mL histamine)	General category of airway reactivity	Number of horses	Mean neutrophil % ± SD	Mean mast cell % ± SD	Mean eosinophil % ± SD
< 8	'Reactive'	17	8.7 ± 4.7	2.8 ± 1.7	4.6 ± 8.6
≥ 8	'Normal'	16	11.1 ± 9.7	3.0 ± 1.8	1.8 ± 2.6

No statistical association was found between independent relative inflammatory cell percentages and general or final category of airway reactivity.

CHAPTER 5. DISCUSSION

In this study we found no association between BALF cytology and airway hyperreactivity (PC_{35} value ≤ 8 mg/mL) using flowmetric plethysmography.

Bronchoalveolar lavage cytology findings

Ninety-five per cent of horses in this study were diagnosed with IAD based on international inclusion criteria outlined by the Havemeyer Foundation Workshop in 2002 (36). This was an unexpected finding. Other studies have reported that IAD is very common in horses in conventional stable environments (55) and an acute increase in airway neutrophils has been reported in response to stabling (27, 30), potentially due to the increase respiratory load of inhaled irritants. The horses in this study were housed outdoors and we thus hypothesise that these inflammatory changes are potentially due to environmental allergens.

Overall the majority of horses (71%) demonstrated a 'mixed' type inflammatory response, the most common of which involved a combination of neutrophil, mast cell and eosinophil increases. Sole increases in individual inflammatory cells alone were less common which resulted in small sample sizes when horses were categorized. There was only one horse with a sole eosinophilic response and only 2 horses were classified as 'normal' in regard to BAL cytology. These small sample sizes in some groups are a source of Type II error in the study and thus the interpretation of statistics should be taken with caution.

Sixty-one per cent (23/38) of horses examined had a pulmonary eosinophilia (eosinophils ≥ 1%), which was also an unexpected finding in our study. Pulmonary eosinophilia has been described in horses with IAD (61, 99) but the significance of this finding remains unclear. In humans with allergic asthma the eosinophil is the hallmark respiratory inflammatory cell (130, 131). Transient pulmonary eosinophilia is reported in horses (132-134). Potential causes of eosinophilic pulmonary inflammation in horses include migrating parasites (*Parascaris equorum*)(134) or lungworm infection (*Dictyocaulus arnfeldi*)(135), idiopathic chronic eosinophilic pneumonia (136) or multisystemic eosinophilic epitheliotropic disease (135). The latter two mentioned conditions are characterised by progressive onset of respiratory clinical signs with pulmonary eosinophilia (135, 136) and can thus be excluded as potential differentials in the horses in our study. In Riihimaki's study (133), transient pulmonary eosinophilia was also an unexpected finding and abated with deworming treatment (137). The possibility that migrating parasites could be a precipitating cause of pulmonary eosinophilia in some of the horses in our study cannot be definitively ruled out. Although faecal parasite testing was not performed, the mature age of the horses makes pulmonary ascariasis unlikely (138). A deworming programme was in place but on further epidemiological investigation the horses were reported to have a history of having grazed land previously inhabited by donkeys and the effectiveness of the anthelmintic programme is not known. Horses with non-patent *Dictyocaulus arnfieldi* infection however are reported to also exhibit a moist non-productive cough (138), in contrast to the horses in our

study which did not demonstrate any clinical signs of respiratory tract disease, thus making lungworm infection less likely.

Our findings would incite some scrutiny in regard to BAL cytological analysis in a population of horses without overt clinical signs of respiratory tract disease such as coughing. The study highlights that what are considered normal values for cell proportions in BALF might vary between different populations of horses. Although the cell population of the equine lower airways is considered uniform and a single BALF sample collected from any lung segment is considered to be representative of the entire lung (89), BAL cytology provides no information on the activity of the observed cells present. There is limited data in human studies but these are in agreement that increased inflammatory cells in the airways are not associated with evidence of cell activation (84). BAL cytology may simply be too crude a measure of airway inflammation.

Pulmonary function testing with histamine bronchoprovocation

Part one of our study investigated the consistency of the Flowmetrics Plethysmography™ system with histamine bronchoprovocation at weekly intervals. Two previous reliability studies using Flowmetrics Plethysmography™ system with histamine bronchoprovocation have been reported in the horse (127, 139). In accordance with these studies sufficient consistency of measurements was demonstrated using the Flowmetrics Plethysmography™ system with histamine bronchoprovocation in our study. An intraclass correlation coefficient (ICC) was used to assess reliability in our study (140).

Nolen-Walston and co-workers (127) used a similar method (Lin's concordance correlation coefficient)(141) to assess reliability in their study. Unfortunately Kuehn et al. (2000) (139) did not report how their reliability statistics were calculated or the type of ICC used in their study. This is considered a fundamental error in reporting statistics (142, 143).

In contrast to these prior reliability studies, we repeated testing at a shorter time interval. Although tachyphylaxis to inhaled histamine has been demonstrated in humans (144) and dogs (145), by extrapolating from these studies we believe it is an unlikely confounding factor in our study. In humans, it is recommended that repeated histamine tests should be separated by more than six hours to avoid tachyphylaxis (144). Testing was performed in our study at weekly intervals.

Interestingly, in our study a higher ICC was calculated for subjective rather than objective data (0.932 v 0.655). Our reasoning for recording subjective data was that we hypothesised that we could determine when a horse had bronchoprovocated based on their clinical findings of increased respiratory rate and depth. A recent study (146) has named the dose of histamine at which inhalation was stopped as the 'histamine challenge score'. The 'histamine challenge score' was found to produce the highest proportion of correctly classified RAO horses based on clinical signs of coughing and nasal discharge in that study.

Airway hyperreactivity ($PC_{35} \leq 8$ mg/mL) was demonstrated in 52% of horses examined in part 2 of our study. Five horses had incomplete data for airway

reactivity due to failure of the pulmonary function testing unit or nasal oedema and were thus omitted from data analysis in part 2 of the study. Nasal oedema also developed in two individual horses on 2 individual occasions in part one of our study, precluding the use of data in these instances. The development of nasal oedema during pulmonary function testing has been reported in other studies (147-149). Nasal oedema can be caused by venous congestion that occurs when horses hold their head down after sedation (150-154). The severity of nasal oedema is correlated with the duration and degree of lowering of the head (151). In order to avoid the development of nasal oedema as a complication of sedation, the horse's heads were placed on a stand and kept upright during pulmonary function testing. Local absorption of histamine can also cause vasodilation and increased permeability of the microvasculature (149, 155). This could possibly have been the cause of nasal oedema in our horses.

Despite evidence that sedation alters lung function in the horse (152, 153, 156-158), pulmonary function testing and histamine bronchoprovocation was unfeasible without the use of sedation in this study. It has been reported in previous studies that alpha-2 agonists do not block the effects of bronchoconstrictive agonists (histamine) and so are appropriate for restraint during bronchoprovocation (101, 117). The recommended approach to use the minimal dose of sedation possible (115, 159) was followed in our study. Detomidine was chosen as a sedative as it has a longer duration of activity than xylazine (160) and thus horses did not require any additional sedation during testing.

Repeated PFT's were performed at the same time of day in part one of the study to avoid the potential influence of daily variations in lung parameters due to fluctuations in inflammatory mediations, endogenous steroids, autonomic, and non-adrenergic non-cholinergeic mechanisms (115, 161). Previous studies have shown that pulmonary function of horses with recurrent airway obstruction (RAO) measured with conventional methods shows daily and/or seasonal variation (161-163). As the horses were housed outdoors, week to week variation in exposure to airway allergens was possible.

Part one of the study was also performed in a temperature controlled air-conditioned room to avoid any potential influence of air humidity or differences in environmental temperature on the testing system or lung function during reliability testing. A study by Onmaz et al (2013) found that neither outdoor nor indoor temperature nor room humidity had a significant effect on airway dynamics (163). Part two of our study was performed in non-temperature controlled room.

Bronchoalveolar lavage cytology and pulmonary function testing findings

Ninety-five percent of horses were diagnosed with inflammatory airway disease based on bronchoalveolar lavage cytology criteria and 52% of horses demonstrated airway hyperreactivity. Despite an obvious overlap in these findings no statistical association between bronchoalveolar lavage cytology and airway reactivity was found.

This phenomenon whereby signs of airway inflammation did not correlate with bronchial hyperresponsiveness has been reported in horses (128, 146, 164) and humans (84, 131, 165, 166). Increased numbers of inflammatory cells in the airways of human athletes are not necessarily associated with any major clinical or functional alterations (84, 165). Furthermore, humans with clinical signs of asthma have also demonstrated negative responses to inhaled histamine (167). The reasons for the finding of normal bronchial responsiveness in humans with current asthma symptoms have not been fully elucidated (168). It appears that airway hyperresponsiveness measured in response to pharmacologic agents such as histamine is different to that measured in response to physical stimuli, such as exercise and osmotic agents (167).

The converse to this phenomenon has also been demonstrated in humans in that a positive airway response to histamine does not infer the presence of inflammatory cells (131, 167, 169). The same finding has been reported in clinically normal horses that have been shown to demonstrate airway hyperresponsiveness, with normal bronchoalveolar lavage cytology (128, 146) or the absence of significant small airway lesions on lung biopsy (148).

Airway responsiveness has been reported to be a transient finding and can change over time with factors such as allergen exposure and viral respiratory tract infections (168-170). This variability of airway responsiveness over time appears to be a major factor in the inconsistency of the relationship between airway hyperresponsiveness and clinical asthma in humans and the same theory is likely applicable to the horse.

Another suggested explanation for this inconsistency is that there may be a variation in the type of inflammation that causes airway hyperresponsiveness in the horse (128). Previous studies in horses have reported an association between a sole increase in mast cell percentage in BALF and airway hyperreactivity (101) or BALF eosinophilia and airway hyperreactivity (99). In contrast to these studies, no association was found between BALF mastocytosis or eosinophilia alone and airway hyperreactivity in our study. Likewise in human studies, the presence of particular inflammatory cell types is not a prerequisite for airway hyperreactivity (166).

In agreement with Pacheco and co-workers (164), no correlation was found between age, BALF cytology and airway reactivity in our population of asymptomatic horses. Further investigation however is warranted in a population of horses with clinical signs of respiratory disease consistent with IAD, such as cough and poor athletic performance.

Conclusion

This study has achieved the primary aim of investigating the association between BALF cytology and PFT with histamine bronchoprovocation in a population of asymptomatic sedentary horses. The secondary aim to assess the reliability of the Open Pleth™ system using sedentary horses in Western Australia was also achieved.

The first hypothesis that there would be a strong association between lower airway inflammation, as demonstrated by BALF cytology, and airway hyperresponsiveness was not fulfilled. Despite an obvious overlap between BALF cytology and airway reactivity, no statistical association was found. Identification of horses with a cytological diagnosis of IAD without AHR was more common than previously reported.

The second hypothesis that flowmetric plethysmography with histamine bronchoprovocation using the Open Pleth™ system is a reliable technique to detect airway hyperresponsiveness was fulfilled. Sufficient reliability was demonstrated. Although the PFT technique appears to be reliable it is possible that both techniques may have limitations with respect to definitive identification of disease.

A limitation to this study was the unexpected high prevalence of IAD, as diagnosed through both BALF cytology and PFT, in a population of outwardly normal sedentary horses. It is possible that unusual environmental triggers may have been present within this population, particularly as it relates to the high incidence of eosinophilia within the group. Investigations in other populations, including those with historical and clinical signs consistent with IAD are warranted.

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